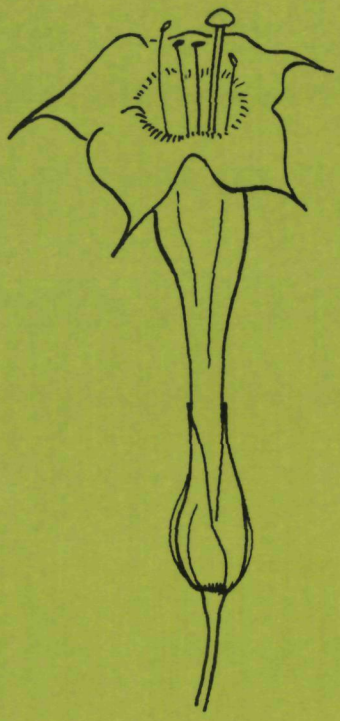
# CYTOKININ REGULATED FLOWER BUD FORMATION IN VITRO IN TOBACCO



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# CYTOKININ REGULATED FLOWER BUD FORMATION IN VITRO IN TOBACCO

# CYTOKININ REGULATED FLOWER BUD FORMATION *IN VITRO* IN TOBACCO

een wetenschappelijke proeve op het gebied van de natuurwetenschappen, in het bijzonder de biologie

PROEFSCHRIFT

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# CHAPTER 1

# Introduction

# INTRODUCTION

## PLANT HORMONES

A plant hormone is an organic compound synthesized in one part of the plant and translocated to another part where, in very low concentrations, it causes a physiological response (Salisbury and Ross, 1985). The response of a certain tissue to a hormone can be promotive, which results in growth and development, or it can inhibit these processes. Although distinct effects can be attributed to specific hormones, growth and development normally are regulated by an interplay between the hormones. Hormones can have identical characteristics, but their various actions are dependent on the kind of tissue and on the plant species. The response of a cell to a hormone is the combined result of sensitivity and the level of the hormone: variations in either could regulate or initiate changes in development itself (Trewavas 1982). Factors that modify sensitivity are subject to continuous variation because plants live in a constantly changing environment and do not posses facilities for maintaining an unchanged internal composition.

Generally, five groups of plant hormones are distinguished: cytokinins, auxins, abscisins, gibberelins, and ethylene. In short the effects of the different hormones will be discussed (for further reading and references on this subject see Wareing 1982 and Leopold 1987).

-- Gibberellins: have an effect on cell division and elongation, dormancy and flowering (Salisbury and Ross 1985).

-- Abscisic acid: influences abscission, dormancy, flowering, and stomatal closure (Salibury and Ross 1985).

-- Ethylene: influences abscission, apical dominance, sex expression, flowering, ripening, wilting and senescence (Abeles 1985).

-- Auxins: promote root elongation at very low concentrations but inhibit this at concentrations higher than 1 $\mu$M (Batra et al. 1975; Scott 1972); they promote adventitious root development on stems (Haissig 1974); support apical dominance (Hillman 1984); stimulate cell division; influence root and shoot growth; and control vascular system differentiation (Bandurski et al. 1987, Cohen and Bandurswki 1982, Nakamura 1986 and Thimann 1988). There are three natural auxins of which indoleacetic acid (IAA) is the most active one. The other two, rather inactive, are

4-chloro-idoleacetic acid and phenylacetic acid (PAA). The synthetic auxins 1-naphthaleneacetic acid (NAA), indolebutyric acid (IBA), and 2,4-dichloro-phenoxyacetic acid are also very active plant growth regulators.

-- Cytokinins: in general, cytokinins are substituted adenine compounds that promote cell division. Three natural cytokinin bases exists: zeatin (Z), dihydrozeatin (DHZ) and isopentenyladenine (IP). Besides these, several synthetic cytokinins are known among which benzyladenine (BA) and kinetin are the most well known. The natural cytokinins are produced in many plant tissues, and exert their effects in many plant parts. In addition to the occurrence in higher plants as free compounds, cytokinins also occur as component nucleosides in tRNA of plants, animals, micro-organisms and plant viral RNA (for a review see Letham et al. 1983).

Since the role of cytokinins in flower bud development is investigated in this thesis the remaining part of this introduction will be restricted to these group of phytohormones.

# CYTOKININ ACTION

## I. *Cytokinin concentration in the plant tissue*

For a good understanding of cytokinin action, it is important to relate the developmental process studied to the cytokinin concentration in the tissue. This endogenous cytokinin concentration can be elevated on three different ways. (A), exogenous application; (B), by tissue wounding which induces cytokinin biosynthesis or: (C) by transformation by Agrobacterium, which introduces the isopentenyltransferase (*ipt*) gene in the tissue.

### I. A. *Exogenous application*

In studying the mechanism of action of the cytokinins, the evoked physiological response in the plant tissue has to be related to the actual endogenous cytokinin concentration. As for cytokinins, these concentrations cannot be determined from the amount of hormone taken up, because of the conversion of the active molecules into derivatives with different activities (Fox et al. 1973, Laloue and Pethe 1982, Laloue et al. 1975, Gawer et al. 1977, Van Staden et al. 1986, Zhang et al. 1987). Metabolic studies resulted in the conclusive identification of cytokinin

metabolites. The most abundant metabolites are the 9–riboside (9R–Cyt), the mono–, –di and triphosphate of the 9–riboside (the nucleotides), the 3–, 7–, and 9–glucoside (3–, 7 – and 9G–Cyt), the O–glucosides of 9R–Cyt and the cytokinin base, the 9–alanine (Letham and Palni 1983, Tao et al. 1983). The glucosides are found to be stable and inactive conjugates. The question whether the free base, the 9R–Cyt or the nucleotides are physiological active has not yet been convincingly answered. Laloue and Pethe (1982) argue that the free base is active; Van Staden et al. (1986) claim activity of the nucleotides, and Letham et al. (1982) found indications for activity of the 9R–Cyt. All results on conjugate activity so far are rather tentative. No recent data are available that elucidate this problem.

Besides a decrease in the active cytokinin concentration through conjugation, a further loss of activity can occur through cytokinin breakdown. Only cytokinins with an unsaturated side chain (Z and IP) are subject to breakdown to adenine or adenosine (Letham and Palni 1983).

## I. B. *Tissue wounding*

Crane and Ross (1986) found that wounding led to increased endogenous cytokinin concentrations. Using this phenomenon the effect of the cytokinin concentration on development could be determined. By various wounding techniques, including rubbing and cutting, growth of cotyledons excized from dark grown cucumber seedlings is promoted (Ross et al. 1983). Giridhar and Thimann (1985) found in oat leaves that wounding delayed senescence. This delay was almost comparable with the delay obtained after external application of cytokinins. Mitchel and Van Staden (1983) indeed found elevated cytokinin levels in wounded potato tubers.

## I. C. *Transformation with the ipt gene*

The soil bacterium *Agrobacterium tumefaciens* contains a plasmid, the Ti plasmid. Part of this plasmid, the T–DNA (T = transfer), can be integrated in the nuclear genome of plant host cells (Hooikaas and Schilperoort 1984). The T–DNA carries two genes that specify the biosynthesis of auxin: T–aux 1 and T–aux 2 gene (encoding for indoleacetic acid) and one gene that specifies the biosynthesis of cytokinin: T–cyt or *ipt*–gene (encoding for isopentenyltransferase; an enzyme that catalyzes a key step in cytokinin biosynthesis) (Morris 1986). The phytohormone

overproduction due to the expression of these genes causes uncontroled cell division of the transformed cells wich leads to the formation of a tumor, named "crown gall" Since (introduction of) uncontroled growth of undifferentiated tissue is in fact oncogenesis, the three genes for phytohormone synthesis are called "onc-genes"

The fact that the T-DNA onc-genes are developmental control genes becomes evident if either the expression of the T-aux or T-cyt gene is blocked If the T-aux is inactivated, only extra synthesis of cytokinins occurs On tobacco this leads to the development of shoots from crown gall tumors Inactivation of the cytokinin onc-gene leads to a high auxin level which results in the formation of roots from the tumor

The T-DNA gene that is associated with cytokinin biosynthesis in plants, the isopentenyltransferase (*ipt*) gene, was introduced into wounded tobacco stems and leaves (Smigocki and Owens 1988) The *ipt* gene was placed under control of promoters which led to largely different amounts of cytokinins in the tissues The obtained results show that a more active *ipt* gene promoter can enhance or change the morphogenetic potential of these transformed plant cells (no roots and more and larger shoots) by increasing their endogenous cytokinin level

## II. *Mode of action of cytokinins*

The cytokinin action can be studied on different levels In this thesis especially the mode of action on the organismal (flower bud formation *in vitro*) and on the molecular level (cytokinin-specific gene expression during bud formation *in vitro*) were studied

## II. A. *Cytokinin action on the organismal level*

In recent research the endogenous cytokinin level was related to the development of many plant organs The cytokinins were found to play an important role in dormancy in potato tubers (Turnbull and Hanke 1985), floral induction (Lejeune et al 1988), adventitious root formation (Bolmark et al 1988), flower bud blasting (Vonk et al 1986), apical dominance, and senescence (Matthysse and Scott 1984)

## II. B. *Cytokinin action on the molecular level*

Very little is known about the action of cytokinins at the molecular level The

general theory about hormones is that the hormone binds to a receptor, which becomes activated and induces the expression of primary target genes (Libbenga et al. 1986, Lewin 1987). The products of these genes can regulate other genes and eventually lead to a cascade of effects.

The first reports about changes at the molecular level during tissue development dealed with the amount of DNA inside the tissues. Generally an age-related decline in DNA in plant cells is reported. Loss of nuclear DNA was described during sequential leaf senescence in ivy (Nagl 1979), peanut, tobacco (Dhillon and Miksche 1981) and rye (Hesemann and Schröder 1982). Tobacco stems are characterized by a progressive loss of DNA with ageing (Wardell and Skoog 1973). In contrast, extra synthesis of repeated DNA sequences occurs in tissues developed in explants of floral branches. In tobacco Wardell and Skoog (1973) showed that stem segments which regenerate flowers in vitro contain more DNA/g fresh weight than explants regenerating vegetative buds; the same was found by Altamura et al. (1987).

Recently, reports of the effect of cytokinins on gene regulation have been published. Cytokinins were found to increase the synthesis of the light harvesting chlorophyll a/b-binding protein in cell cultures of tobacco (Axelos et al. 1984, Teyssendier et al. 1985) and Lemna gibba (Flores and Tobin 1986). A reduction of the mRNA level of ß-1,3glucanase and chitinase under the influence of applied cytokinin was found in tobacco (Mohnen et al. 1985, Shinshi et al. 1987). In contrast, in tobacco shoots with an elevated cytokinin level due to transformation with a vector containing an active isopentenyl transferase gene of Agrobacterium, or due to application of cytokinin in a culture medium, an increase in the levels of 5 mRNA species related to defense-related proteins occurred (Memelink et al. 1987). Four of these genes coded for extensin, chitinase, pathenogenesis-related protein 1 (PR 1), PR 1 like. The nature of the gene product of the fifth gene is still unknown.

# CULTURE *IN VITRO*

## *Tissue culture*

Processes of growth and differentiation normally proceed in organized groups of cells in a relatively small part of the plant. An advantage of tissue culture is that processes can be made to occur on a large scale in small parts of cultured tissue

(organ fragments). Also the synchronicity of the developmental processes can be improved. Finally, in tissue culture there is no interference of (unknown) regulatory factors of the plant with the hormonal regulation of the regeneration processes.

Although culture *in vitro* offers many advantages in studying growth and development, the knowledge obtained from tissue culture has to be extrapolated to the situation in the intact plant, which should be done with great care. The disadvantage is that in tissue culture some aspects are not comparable to that in the whole plant. In tissue culture parts of plant tissues are cultured that consist of differentiated cells. During culture, these cells dedifferentiate. Then the new cells differentiate into new cell types, forming new tissues and organs (Bhojwani and Razdan 1983). In the intact plant, organ formation starts via differentiated cells that are produced by already existing meristems, such as those in root and shoot tip (Torrey 1966). The regeneration in tissue culture is much more like adventitious root or shoot formation *in vivo*, where organs develop also from differentiated cells and not from meristems. Another difference with the *in vivo* situation is, that cells in the plant may never be exposed to such large changes in hormone concentration as those used to evoke effects in cells cultured *in vitro*.

In tissue culture of tobacco the type of organ formed depends on the plant part cultured. On stem segments the concentrations and ratios of auxins and cytokinins determine the induction and frequency of roots and/or shoots (Skoog and Miller 1957). Tran Thanh Van (1973, 1974, 1981) found that the formation of flower buds and vegetative buds on thin-layer explants depended on the part of the plant from which the explants were taken. On pedicel explants only flower buds were formed. The floral ramnifications formed both flower vegetative buds, depending on the concentration and type of cytokinin used. Stem explants solely formed vegetative buds. In this thesis the pedicel system originally developed by Tran Thanh Van (1973) and modified by Van den Ende et al. (1984) served as a model system for the study of the role of cytokinins during bud formation. The system is very well suited, since (A) the explants originally only consist of the epidermis and a few layers of cortical cells (Wilms 1988), (B) flower buds regenerate within two weeks without intervening callus formation, (C) large numbers of flower buds are formed, and (D) relatively large proportions of (cortex) cells are presumably involved in the regeneration process. In this pedicel strip system, an auxin and a cytokinin apparently regulate the level and pattern of gene expression responsible for morphological development.

# TOPICS UNDER INVESTIGATION

This thesis is divided in three main parts. The first part concerns the relation between flower bud formation and cytokinin uptake and metabolism (chapter 2,3,4,5). The second part deals with the role of the concentration of endogenous synthesized cytokinins on bud formation (chapter 6). The third part is about the relation between the cytokinin concentration in the explants and the expression of cytokinin– related cDNA copies during bud formation (chapter 7).

Chapter 1: introduction.

Part I: Uptake and metabolism of cytokinins during flower bud formation *in vitro*

Chapter 2: identification of the metabolites formed from BA; uptake and metabolism of BA at different medium concentrations, both at inductive and non–inductive level for flower bud formation.

Chapter 3: effect of six cytokinins on flower bud formation; identification of the active cytokinin compound by using relatively inactive cytokinin as a competitive inhibitor of the metabolism of a very active one.

Chapter 4: a study of the fate of BA after uptake; determination of the role of the different metabolites by investigation of their interconversions.

Chapter 5: assessment of the dose of two cytokinins that induce maximal flower bud formation at the same medium concentration, but after different initiation periods. The role of cytokinin uptake and metabolism will be discussed in the estimation of the activity of the cytokinins.

Part II: the role of endogenously synthesized cytokinins on flower bud formation *in vitro*

Chapter 6: assessment of the endogenous cytokinin levels during flower bud formation; the effect of wounding, exogenous applied cytokinin, and auxin on the endogenous cytokinin level.

Part III: cytokinin–related gene expression during flower bud formation *in vitro*

Chapter 7: the effect of the exogenous applied cytokinin and auxin, and endogenously synthesized cytokinin levels on the gene expression of cytokinin–specific cDNA copies during flower bud formation.

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# PART 1: UPTAKE AND METABOLISM OF CYTOKININS DURING FLOWER BUD FORMATION IN VITRO

# CHAPTER 2

## Uptake and metabolism of benzyladenine in the early stage of flower bud formation in vitro in tobacco

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Physiologia Plantarum (1988) 74: 113–118

# Uptake and metabolism of benzyladenine in the early stage of flower bud development in vitro in tobacco


## Abstract

Benzyladenine (BA) was found to regulate the number of flower buds regenerated *in vitro* from pedicel tissue of tobacco. Flower bud induction is particularly sensitive to BA levels in the range of 0.45 to 1.0 $\mu$M, where a twofold increase in concentration causes a threefold rise in the number of buds. When tissues were fed radioactive BA for 24 h, only 9–12 % of the counts were recovered in the original compound. The rest was present in metabolites tentatively identified as the mono–, di– and tri–ribotides, the 7– and 9–glucosides, and the 9–riboside of BA. The amount of growth regulator taken up and the quantities of BA and its metabolites in the explants were all linearly related to the concentration of the medium. The internal BA concentration was ca 60 % of the level in the medium after 24 h. When the concentration in the medium was raised, relatively more BA remained in the non–conjugated form. However, this change in the equilibrium between BA and the conjugates is too small to account for the steep rise in the curve representing concentration vs effect between 0.45 and 1.0 $\mu$M.



*Key words* – benzyladenine, cytokinin metabolism, flower bud development, *Nicotiana tabacum*, tissue culture, tobacco.


*Abbreviations* – Ade, adenine; Ado, adenosine; 9Ala–BA, L–beta–(6–benzyladenine–9–yl)alanine; BA, n6–benzyladenine; 3G–BA, 3–beta–D–glucopyranosyl–BA; 7G–BA, 7–beta–D–glucopyranosyl–BA; 9G–BA, 9–beta–D–glucopyranosyl–BA; 9R–BA, 9–beta–D–ribofuranosyl–BA; HPLC, high performance liquid chromatography; NAA, naphthaleneacetic acid; TEA, tri–ethylamine; TLC, thin–layer chromatography.

## Introduction

The regeneration of organs from plant tissues *in vitro* opens the possibility to study

differentiation in small explants Thus the qualitative and quantitative regulation by auxins and cytokinins of developmental processes, spatially isolated from the whole plant, can be studied. It is known that in tobacco the concentrations and ratios of these plant growth regulators determine the induction and frequency of roots and/or shoots (Skoog and Miller 1957) or flower buds (Tran Thanh Van 1981) A model system for the study of hormone action in differentiation is the formation of flower buds on epidermal strips from pedicels of tobacco This system was originally developed by Tran Thanh Van (1973a b) and modified by Van den Ende et al (1984) The explants originally consist of the epidermis and a few layers of cortical cells Flower buds regenerate within two weeks without intervening callus formation Because large numbers of flower buds are formed, relatively large proportions of cells are presumably involved in the regeneration process

In this pedicel strip system, naphthaleneacetic acid (NAA) and BA apparently regulate the level and pattern of gene expression responsible for morphological development In attempting to understand the mechanism of action of these plant growth regulators, it is important to relate the number of flower buds produced to the actual endogenous auxin and cytokinin levels As for cytokinins, these levels cannot be determined from the amounts of growth regulators taken up because of the conversion of the active molecules into derivatives of different activity (Fox et al 1973, Laloue et al 1975, Gawer et al 1977, Van Staden et al 1986, Zhang et al. 1987)

Benzyladenine is known to be metabolized to a number of conjugates (Letham and Palni 1983) In radish cotyledons, the principal metabolites immediately after uptake are the mono-, di- and triphosphates of benzyladenosine After a few hours and depending on the tissue the 7-or 9- glucoside of BA is the most abundant metabolite The physiological role of these metabolites has not fully been elucidated Generally the 7- and 9-glucosides are considered stable and inactive (Laloue et al 1975 Laloue 1977, Letham et al 1982) In contrast, the nucleotides and 9R-BA may themselves be active (Letham and Gollnow 1985) or easily converted into the active regulator (Laloue and Pethe 1982)

In this paper we describe the role of BA in flower bud formation *in vitro* In particular, the uptake and conversion of BA was quantified at effective and ineffective (medium) concentrations The calculated internal levels of BA and its metabolites were related to the extent of flower bud regeneration

# Materials and methods

## *Culture in vitro*

Flowers of tobacco (Nicotiana tabacum L. cv. Samsun) were picked with pedicels attached at anthesis when the inflorescence was in stage IV (Croes et al. 1985). Small explants (8 x 1 mm) were cut from the flower stalks and cultured on the medium of Murashige and Skoog (1962) with 125 mM glucose and 1% agar, according to Van den Ende et al. (1984). NAA and BA were added at various concentrations as indicated for each experiment. Buds were counted 14 days after the onset of culture. The data were subjected to logarithmic transformation and analysis of variance. Least significant difference was calculated at $p = 0.05$. In most experiments [2,8-$^3$H] BA (specific activity 15.5 GBq mmol$^{-1}$, Amersham, UK) was included for quantification of cytokinin uptake and conversion.

## *Extraction and HPLC analysis*

For the identification of the more polar metabolites (nucleotides) of BA, groups of 10 explants were cultured on [$^3$H] BA. For a characterisation of the more non-polar metabolites (glucosides and the riboside of BAP), 1000 explants were cultured on [$^3$H] BA. In either case, the procedure of Laloue and Pethe (1982) was used with modifications. Briefly, the explants were homogenised after incubation in modified Bieleski fixative (Laloue and Pethe 1982). After standing overnight at 4 °C, the homogenate was centrifuged and the pH of the supernatant adjusted to 8 with $NH_4OH$. The supernatant was then dried under nitrogen at 40 °C. For the purification of BA and 7G-BA, the residue was dissolved in 35% ethanol and passed through an LH-20 column (Pharmacia, Sweden). The column was eluted with 35% ethanol and fractions containing radioactivity were pooled and lyophilised. The residue was dissolved in 1 mM $KH_2PO_4$, pH 5, and purified by elution from an ion-exchange HPLC column (250 mm, 3 mm, Partisil 10 SAX, Whatman, UK). The sample was eluted with 1 mM $KH_2PO_4$ in 7% methanol at a flow rate of 1.5 ml min$^{-1}$.

For an examination of the non-polar metabolites, the void volume was collected and analysed on a RP 18 HPLC column (250 mm, 3 mm, Lichrosorb, Merck, FRG). The mobile phase was a linear gradient of 0 to 100% methanol buffered with 20 mM acetic acid-TEA buffer at pH 5.6. The elution rate was 1 ml

min$^{-1}$ The absorbance of the eluate was measured at 268 nm For an analysis of the more polar BA metabolites the residue after drying under nitrogen, was redissolved in water at pH 8 adjusted with $NH_4OH$ The extract was partitioned 3 times between water and 3 volumes of ethylacetate The water phase was dried under nitrogen at 40 °C The residue was dissolved in 1 mM $KH_2PO_4$ and examined on the ion-exchange HPLC column The sample was eluted for 5 min with 1 mM $KH_2PO_4$ in 7% methanol at pH 5 adjusted with TEA, followed by a convex gradient of 1 to 750 mM $KH_2PO_4$ in 7 % methanol (pH 5 6), at a flow rate of 1 5 ml min$^{-1}$ Radioactivity was measured by liquid scintillation counting after mixing 0 5 ml of 1 min-fractions with 4 ml Lumagel (Lumac)

### *Enzymatic hydrolysis of the BA-nucleotides*

The fractions with retention times on the ion exchange column similar to AMP, ADP and ATP were incubated for 1 h with 0 5 units apyrase (EC 3 6 1 5 , Sigma) in 150 l of 20 mM acetate buffer pH 6 5 and after raising the pH to 10 5 with 1M KOH, with 2 units alkaline phosphatase (EC 3 1 3 1, Sigma) for 1 h After addition of 1 ml ethanol, centrifugation and evaporation of the supernatant, the samples were again analysed by HPLC as described above

### *Separation of BA-metabolites by TLC*

Groups of 10 explants were extracted as described, but after drying under nitrogen the residue was dissolved in 50% ethanol The extracts were chromatographed according to Tao et al (1983) together with standard 7G-BA, 9G-BA, 3G-BA, 9R-BA, 9Ala-BA, AMP, Ade, Ado and BA on silica gel 60 $PF_{254}$ plates Merck, FRG) The glucoside and alanine derivatives were a generous gift of Dr DS Letham The solvents used were n-butanol 14 M $NH_4OH$ water (6 1 2, upper phase) in the first, and n-butanol acetic acid water (12 3 5) in the second dimension To quantify the radioactivity on the TLC plates, the UV absorbing spots were scraped off and extracted with 0 5 ml water for 2 h After adding 4 ml Lumagel, radioactivity was measured by liquid scintillation counting In some experiments separation on one-dimensional TLC proved to be sufficient

## Results

### *Effect of auxin and cytokinin on flower bud formation*

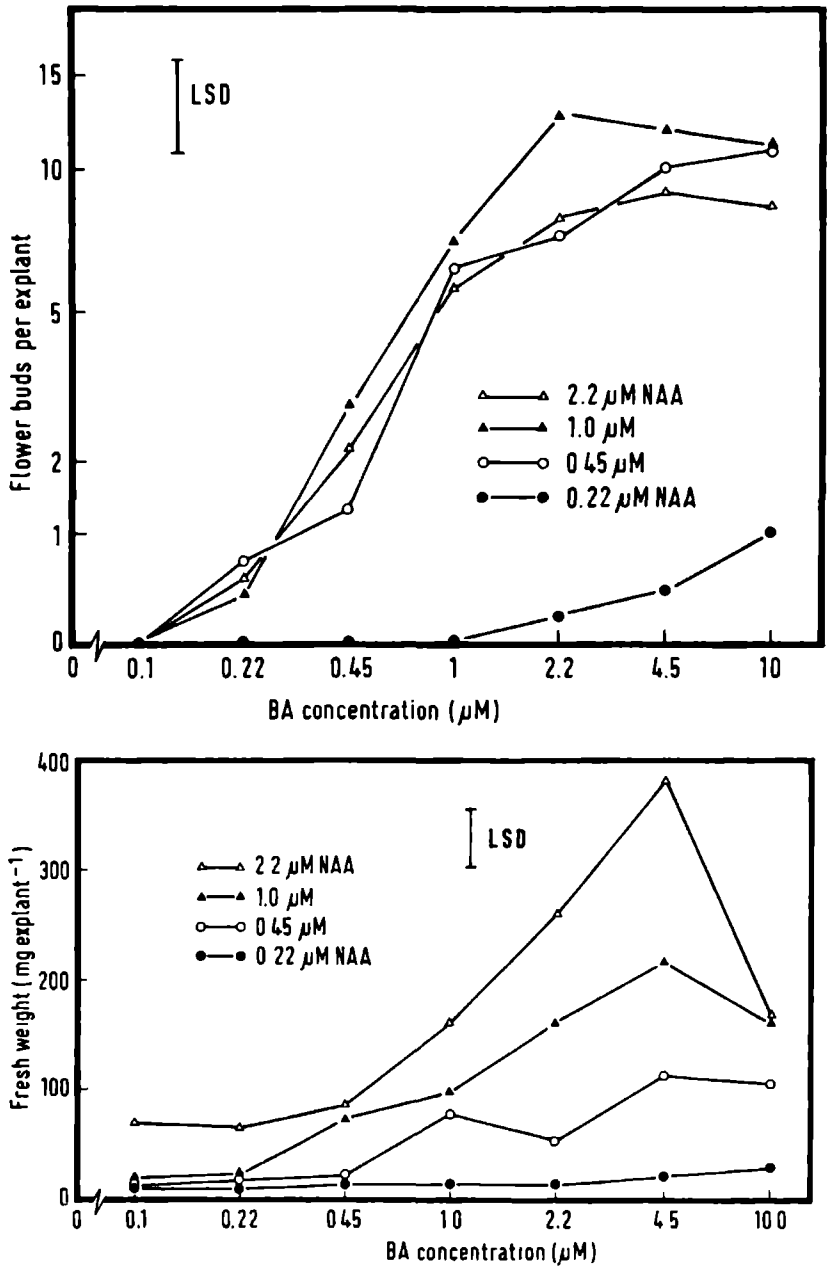


Fig.1A,B. Effect of BA and NAA on flower bud formation in vitro (A) and fresh weight (B). BA concentration was varied at four levels of NAA: ● 0 22μM ○ 0 45μM, ▲ 1μM, △ 2 2μM Buds were counted 14 days after onset of the culture The numbers of flower buds were logarithmically transformed 20 explants were used per treatment Least significant difference (LSD) at $p = 0.05$

The effect of the BA concentration on flower bud formation *in vitro* is presented in Fig. 1A. There is a steep rise in the number of flower buds over the range of concentrations from 0.45 to 1 $\mu$M. Higher concentrations did not lead to a further increase in flower bud number ($P < 0.05$). At very high BA concentrations (i.e. 10$\mu$ M or more) the buds were smaller and the flower parts less developed. Varying the concentration of NAA over the range of 0.45 to 2.2 $\mu$M did not affect the slope of the concentration vs effect curve for BA.

The increase in fresh weight (Fig. 1B) reflects callus formation on the explants. At low concentrations of BA (0.45 $\mu$M or less) BA has little or no effect on the callus formation. At BA concentrations between 0.45 and 4.5 $\mu$M the action of BA and NAA is synergistic. A BA concentration of 10 $\mu$M is supraoptimal especially at high NAA concentrations.

## *Identification of metabolites of BA by two-dimensional TLC*

To study the fate of BA once taken up, an extract of explants incubated on [$^3$H] BA was subjected to two-dimensional TLC. Radioactive spots were found at the positions of 7G-BA, the nucleotides and BA itself A few counts co-chromatographed with 9R-BA and 9G-BA. No radioactivity was found in the 9-Ala-BA, 3G-BA,

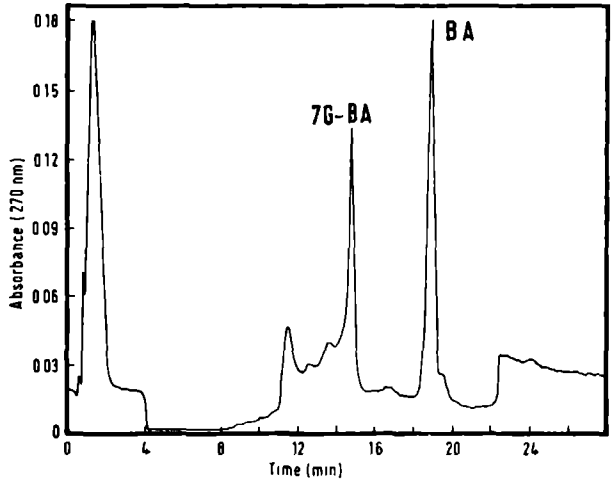


Fig. 2. HPLC separation of the relatively non-polar metabolites of BA Explants of flower stalks were incubated on 1 $\mu$M BA and 1 $\mu$M NAA for 24 h After extraction and purification the non-polar metabolites were separated with reversed-phase HPLC

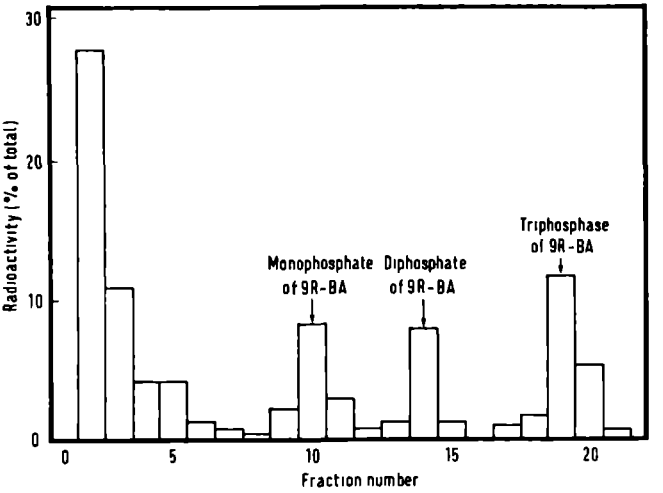


Fig 3. HPLC separation of the relatively polar metabolites of BA after a pulse chase incubation (1 h pulse 3 h chase) The BA metabolites were extracted as described in Materials and methods and analysed on ion exchange HPLC

Ade or Ado spots.

## *Identification of metabolites of BA by HPLC*

The relatively non-polar BA metabolites were more definitely identified by HPLC. One metabolite was detected (Fig. 2) which co-chromatographed with standard 7G-BA and had an identical UV-spectrum

The relatively polar metabolites were characterized in a pulse-chase experiment after incubation on 1 . M NAA and BA for 1 h in the presence of [$^3$H] BA and for 3 h in the absence of [$^3$H] BA (Fig 3). The retention times of the three metabolites were close to those of AMP, ADP and ATP, respectively. Digestion with apyrase and

Tab 1 Metabolites formed from BA after 24 h of incubation on MS medium with 1 $\mu$M NAA and 1 $\mu$M [$^3$H]BA The extract of 10 explants was co-chromatographed on two-dimensional TLC with standards of BA metabolites After visualizing the standards under UV-light the spots were scraped off and radioactivity was determined

| Compound | Radioactivity, % |
|---|---|
| 3G-BA | -- |
| 7G-BA | 79,4 |
| 9G-BA | 1.0 |
| 9Ala-BA | -- |
| Nucleotides | 9.0 |
| 9R-BA | 1.5 |
| BA | 9.1 |
| Ade | -- |
| Ado | -- |

alkaline phosphatase resulted in the displacement of the metabolites from their respective positions into the void volume. This is taken as evidence that the metabolites are 9R–BA 5' mono–, di– and triphosphate.

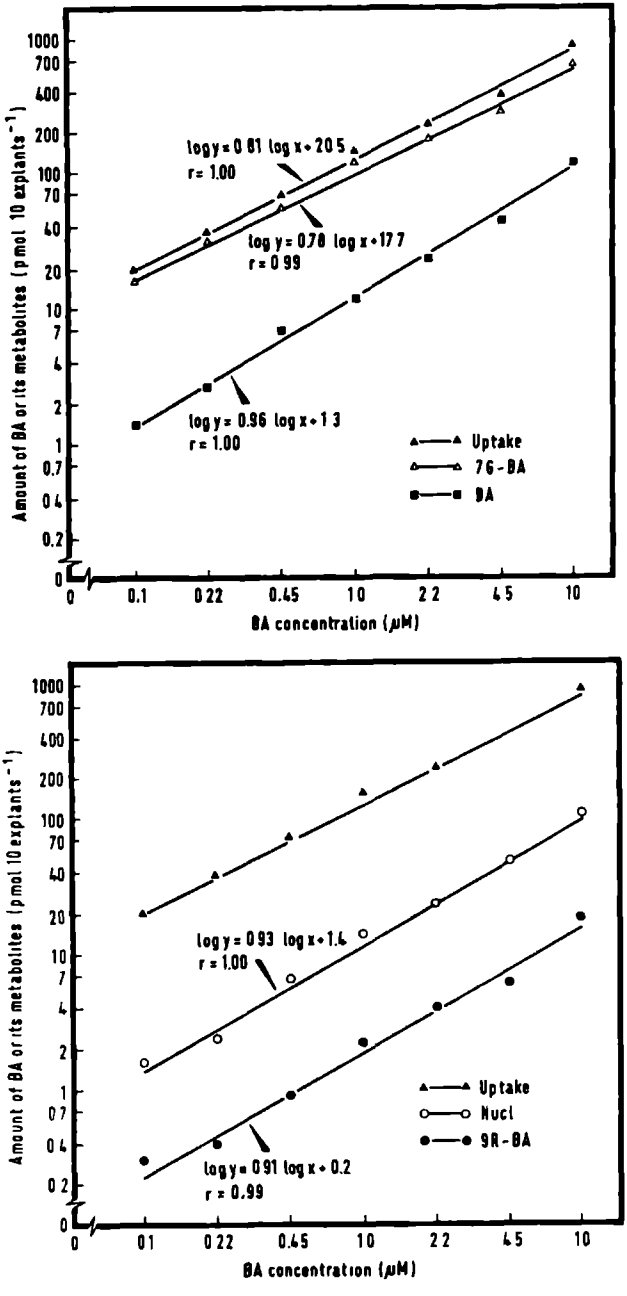


Fig. 4A,B. Uptake of BA and distribution of its metabolites after 24 h of culture in relation to the concentration of BA in the medium. A, Uptake, 7G-BA and BA. B, Uptake, nucleotides and 9R-BA The equations of the linear regression lines and the correlation coefficients (r) are shown.

The radioactivity found in TLC spots after an incubation of 24 h on [$^3$H] BA can now be attributed to individual compounds. 7G–BA was found to be the most abundant metabolite (Tab. 1). Of the other glucosides, only 9G–BA was present in a very small quantity (approximately 1%). The amount of the 9R–BA was also about 1%. The nucleotides and the free base each represented about 4–6 % of the fraction.

With the exception of 7G–BA and 9G–BA, all metabolites (Tab. 1) are easily separated in the first dimension. Therefore, one–dimensional TLC with n–butanol:14 M $NH_4OH$:water (6:1:2, upper phase) as the solvent was used throughout. As a consequence, the radioactivity in 9G–BA (ca 1 %) was not separated from 7G–BA.

## *Cytokinin metabolism during early flower bud formation*

To investigate the relation between the internal concentrations of the various metabolites of exogenously supplied BA on one hand, and flower bud formation and callus initiation on the other, the uptake and conversion at physiologically effective concentrations of BA were investigated at a constant level of 1 $\mu$M NAA. When the BA concentration in the culture medium was raised from 0.1 to 10 $\mu$M, the uptake of BA increased linearly ca fourtyfold (Fig. 4). The amounts of BA and all metabolites present in the explants after 24 h are linearly related to the concentration of BA in the medium. However, the slopes of the regression lines of BA and of the nucleotides differed significantly ($p<0.05$) from the slope of the

Tab. 2. Concentration of BA and its metabolites in explants, after 24 h incubation on culture medium with 1$\mu$M NAA and 0.45 or 1$\mu$M [$^3$H]BA. The values (mmol kg$^{-1}$) are means of 2 duplicate experiments. Explant fresh weight was 2 mg. 'Uptake' refers to the summed concentrations of BA and its metabolites.

| Concentration of [$^3$H]BA in the medium, $\mu$mol (kg FW)$^{-1}$ | internal concentrations, $\mu$mol (kg FW)$^{-1}$ | | | | |
|---|---|---|---|---|---|
| | BA | Nucl. | 9R–BA | 7G–BA | 'Uptake' |
| 0.45 | 0.30 | 0.30 | 0.05 | 2.9 | 3.6 |
| 1.0 | 0.61 | 0.60 | 0.09 | 5.3 | 6.6 |

uptake line. This means that changes in the concentration of BA in the culture medium influence the distribution of counts over BA and its metabolites in the explants. The internal level of free growth regulator after 24 h culture is about 60% of that of the medium (Tab. 2).

# Discussion

The rate of uptake of BA during the first 24 h of flower bud regeneration was about 20 times lower than that of NAA , the auxin used in this system (Barendse et al. 1987, Smulders et al. 1988). Most of the BA taken up was converted to 7G–BA whereas BA nucleotides and 9R–BA were the minor components formed (Tab. 1). Comparable results have been published before (Fox et al. 1973, Gawer et al. 1977, Van Staden et al. 1986). Although side chain cleavage does occur at low rate in some tobacco tissues (Gawer et al. 1977, Laloue and Pethe 1982) there is no indication for this type of BA breakdown in pedicel explants. Although the rate of uptake would have led to a BA concentration that is higher in the explants than in the medium after 24 h, the actual internal concentration was 60 % of the medium concentration due to the extent of this conversion (Tab. 2). In contrast, the level of NAA at the same time is 16 times higher than that in the medium (Barendse et al. 1987). This means that the auxin to cytokinin ratio inside the explants differs very much from that in the external medium. Our data are not sufficient to determine which conjugates play a role in the induction of bud formation, since the levels of all these compounds are positively correlated with bud number (Figs. 1 and 4). If the 7G–BA is indeed inactive (Letham and Palni 1983), the question remains whether the nucleotides and 9R–BA have a biological function. No convincing evidence exists on this point (Laloue and Pethe 1982, Letham and Palni 1983, Van Staden et al 1986). One approach to the problem would be to compare the biological activities of BA and 9R–BA. This is currently under investigation.

Over the range of 0.45 to 1 $\mu$M, bud number increases 3 to 4–fold in response to an only 2–fold rise in BA concentration. One explanation could be that at the higher concentration a lower amount of the BA taken up is inactivated by conversion to 7G–BA. This would result in a disproportional increase in the internal levels of free BA, nucleotides and 9R–BA. Such a shift in metabolism was found (Fig. 4) but the effect is too small (Tab. 2) to account for the steepness of the concentration vs effect curve (Fig. 1). The shift is nevertheless remarkable since no such changes in metabolism in relation to BA–induced developmental processes

have been observed by others (Letham et al. 1982, Letham and Gollnow 1985 and Zhang et al. 1987). The relation between BA concentration and bud number is barely affected by the NAA concentration, provided the auxin is present above a minimum level. The absence of interaction between the two growth regulators suggests that they affect different processes at the molecular level. After buds are initiated callus is formed on the explants (Smulders et al. 1988). The BA concentration dependence of this process markedly differs from that for bud generation. This implies that callus growth and bud development although both dependent on BA concentration, are separately regulated.

The experiments described were focussed on the first 24 h of flower bud initiation. Studies on BA metabolism during the complete 4-day period of bud induction (Van den Ende et al. 1984) are underway.

In conclusion, flower bud regeneration on tobacco explants was found to be regulated by internal concentrations of BA which are low when compared to the effective auxin concentrations although the optimal medium concentrations are similar. Our results do not exclude the possibility that one or more of the products of the extensive BA conjugation also play a role in development.

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# CHAPTER 3

## Cytokinins and flower bud formation in vitro in tobacco. Role of the metabolites

Wim Mvan der krieken, Rene MJ Smulders, Anton F Croes and George J Wullems (1990) Plant Physiology in press.

# Cytokinins and Flower Bud Formation *in vitro* in Tobacco. Role of the Metabolites


## ABSTRACT

Explants from flower stalks of *Nicotiana tabacum* L were cultured on different cytokinins to induce flower bud formation All cytokinins tested except zeatin and zeatin–riboside induced the same maximal number of flower buds Benzyladenine, benzyladenosine and dihydrozeatin were the most active compounds whereas isopentenyladenosine and isopentenyladenine acted at a twentyfold higher concentration These data suggest that the active cytokinins bind to the same receptor with different affinity The presence of benzyladenine in the medium was only necessary during the first two days of culture (initiation period) The equilibrium between benzyladenine and its conjugates (the riboside, glucoside and nucleotides) after a four–day pulse was independent of the benzyladenine concentration whether it was inductive or non–inductive for bud formation The level of all derivatives was proportional to the benzyladenine concentration in the medium

Isopentenyladenine was used as a competitive inhibitor of benzyladenine conjugation. Isopentenyladenine concentrations that were too low for bud formation, led to a synergistic increase in bud number when applied together with benzyladenine Isopentenyladenine decreased benzyladenine uptake and conjugation In spite of the lower uptake, the concentration of free benzyladenine inside the explants was higher in the presence of isopentenyladenine than in its absence whereas the concentration of the 7–glucoside of benzyladenine was lower. It was concluded that the free cytokinin base is the main active compound


[2] Abbreviations BA, 6–benzyladenine, IP, 6–(–2–isopentenyl)adenine, Z, zeatin; NAA, 1–naphthaleneacetic acid, DHZR, DL–dihydrozeatin–riboside, ZR, zeatin–riboside, 7G–BA, 7–ß–D–glucopyranosyl–BA, 9R–BA, 9–ß–D–ribofuranosyl–BA, Ade, adenine, Ado, adenosine, DHZ, dihydrozeatin, IPA, 6–(–2–isopentenyladenosine, LSD, least significant difference

# INTRODUCTION

The regeneration of organs in small explants from plant tissues offers the possibility to study differentiation on tissues spacially isolated from the whole plant. In this way the qualitative and quantitative response to auxins and cytokinins involved in the regulation of developmental processes can be studied. In tobacco both the effects of the concentrations and of the ratios of these plant growth regulators on the induction and frequency of roots and/or shoots (16) or flower buds (20) have been described. In attempting to understand the mechanism of action of these plant growth regulators, it is important to relate the number of regenerated organs to the actual endogenous auxin and cytokinin concentrations. These concentrations cannot be determined from the amount of growth regulator taken up because of the conversion of the biologically active molecules into derivatives of unknown activity or no activity at all (1, 6, 8, 9, 12, 23, 24). The cytokinin conjugates formed are glucosides, ribosides and ribotides. Generally, the glucosides are considered stable and inactive. In contrast, the free base and perhaps also the ribosides and ribotides are active (11, 13).

Little research has been performed on cytokinin metabolism in relation to developmental processes in plants. Van der Krieken et al. (22) studied the effect of BA on its metabolism in the early stages of in vitro flower bud formation in tobacco. A more than ten-fold stimulation of bud regeneration was observed over a concentration interval of one decade. Coinciding with the increase in buds at elevated BA concentrations a small but significant change in favor of the free hormone was found in the equilibrium between BA and its conjugates. This change however was too small to account for the unexpectedly steep rise in the concentration vs effect curve. Comparable results have been obtained in other developmental processes. Zhang et al. (24) studied BA metabolism in presenescent and early senescent soybean leaves. No specific differences in metabolism were present in the two types of leaves.

A significant shift in the abundancy of the free hormone and its conjugates would lead to dissimilar changes in the concentrations of these compounds. Such changes if accompanied by a modification of the physiological response, could give information about the identity of the active molecules. One approach to influence the conjugation equilibrium would be to inhibit cytokinin conversion by competitive inhibition. A cytokinin with low physiological activity would be an ideal competitor.

The thin–layer tissue culture system, originally developed by Tran Thanh Van (18), is very well suited to study cytokinin metabolism in relation to flower bud formation. The explants consist of the epidermis and a few layers of cortical cells. Flower buds regenerate within two weeks without intervening callus formation. As a response to application of auxins and cytokinins large numbers of buds are formed and a relatively large proportion of the cells is involved in the regeneration process. The explants cut from flower stalks (21) consist of the epidermis and a few layers of cortical cells. In this system not only the ratio between auxin and cytokinin , but also the kind of cytokinin used was found to be decisive for the nature of the organs regenerated (19). The synthetic cytokinins BA and kinetin were found to induce flower buds whereas the natural isopentenyladenine and zeatin were inactive. In contrast, Heylen and Vendrig (10), who tested these two natural cytokinins at low concentrations, found regeneration of only a few flower buds in their tobacco culture system.

In this paper we describe the activities of four cytokinins and their ribosides with respect to flower bud formation over a wide range of concentrations. To gain insight in the activity of the conjugates we also studied the conjugation and the physiological activity of a highly active cytokinin in the presence of a cytokinin with little activity which could act as a competitive inhibitor.

# MATERIALS AND METHODS

## Culture in vitro.

Flowers of tobacco (*Nicotiana tabacum* L. *cv. Samsun)* with pedicels attached were picked at anthesis when the inflorescence was in stage IV (7). Small explants (8 x 1 mm) were cut from the flower stalks and cultured on Murashige and Skoog (15) medium with 125 mM glucose and 1% (w/v) agar, according to Van den Ende et al. (21). The NAA concentration was 1 $\mu$M if not indicated otherwise. The cytokinins were purchased from Sigma. DHZR[2] was a racemic mixture of the enantiomers, ZR was for 90% in the trans and 10% in the cis form. The various cytokinins were added at a range of concentrations as indicated for each experiment. Buds were counted 14 days after the onset of culture. The data were subjected to logarithmic transformation and analysis of variance (21). Least significant differences were calculated at $p = 0.05$. In some experiments [2,8–$^3$H] BA (specific activity 15.5 TBq mol$^{-1}$, Amersham, UK) or [2,8–$^3$H] IP (specific activity

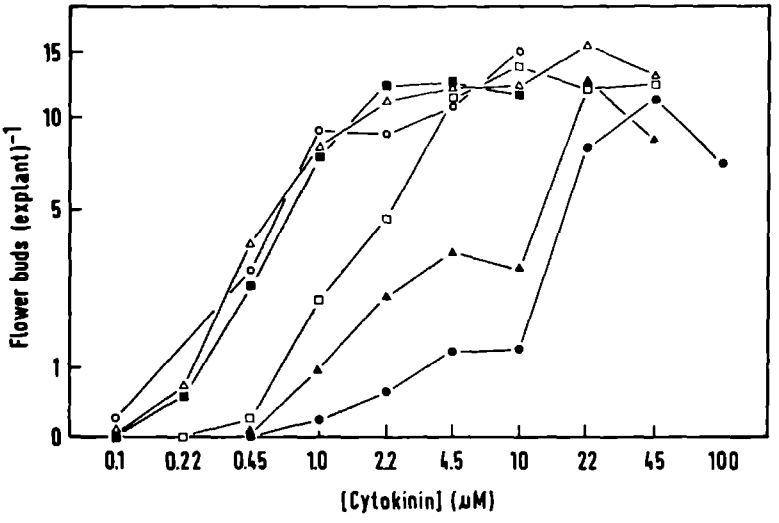


Fig 1. Effect of six cytokinins on flower bud formation in vitro Explants were incubated for 14 d on Murashige and Skoog *medium*. △ BA, ○ ,9R-BA, ■ DHZ, □ ,DHZR, ▲ ,IPA, ● ,IP 20 explants were used per treatment Vertical bar LSD at $p = 0.05$

7.4 TBq mol$^{-1}$) obtained through courtesy of Dr DS Letham was included for quantification of cytokinin uptake and conversion.

## Cytokinin extraction and TLC analysis.

For extraction the method of Laloue and Pethe was used with some modifications. Briefly, the explants were homogenized in modified Bieleski fixative (11). After standing overnight at 4°C, the homogenate was centrifuged and the supernatant dried under nitrogen at 40°C. The residue was then dissolved in 50% ethanol. The extracts were chromatographed by TLC on silica gel 60 $PF_{254}$ plates Merck, FRG) according to Tao et al. (17). The solvent used was *n*-butanol: 14 M $NH_4OH$: water (6:1:2, v/v/v, upper phase). Standard 7G-BA, 9R-BA, AMP, Ade, Ado and BA were chromatographed with the extracts In this TLC system the conjugates of BA banded at the same $R_f$ values as the corresponding conjugates of IP. To quantify the radioactivity in BA or IP derivatives on the TLC plates, the UV-absorbing spots were scraped off into scintillation vials and extracted with 0.5 mL water for 2 h. After adding 4 mL Lumagel (Lumac, Schaesberg, Netherlands) the radioactivity was measured by liquid scintillation counting

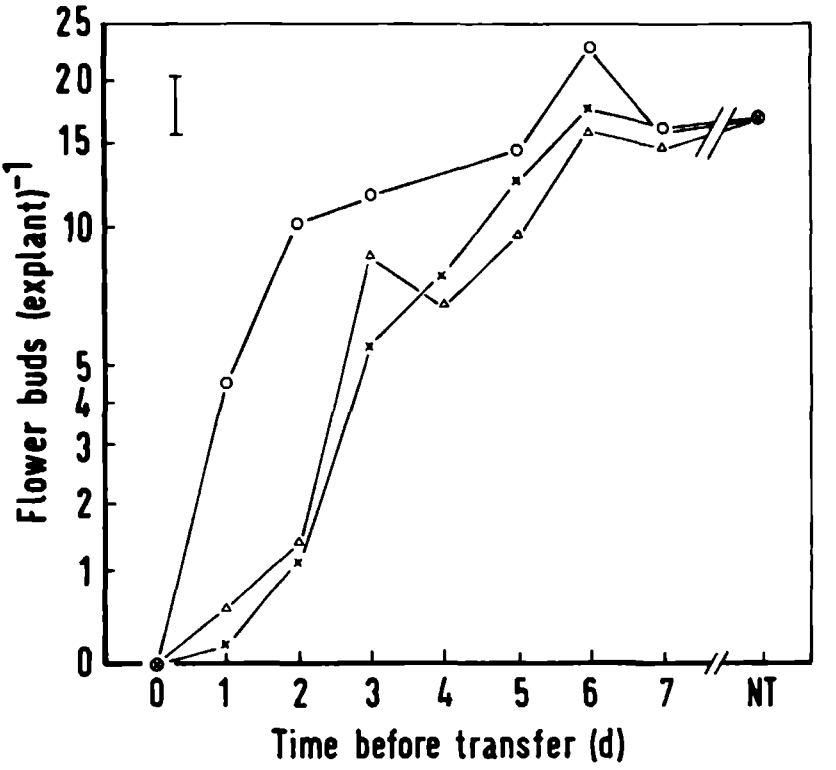


Fig 2 Period during which the presence of BA and NAA is necessary to induce bud formation Explants were first cultured on complete medium At the times indicated, they were shifted to media without BA ○ without NAA △, or without BA and NAA (x) Vertical bar LSD at $\rho = 0.05$

# RESULTS

## Effect of cytokinin on flower bud formation.

The effects of BA, 9R–BA, DHZ, DHZR, IP and IPA on flower bud formation in vitro are presented in Fig. 1. There are clear differences in the sensitivity of the tissue to the different hormones although the maximum bud numbers are equal BA, 9R–BA and DHZ proved to be the most active cytokinins. IP and IPA are less active and form flower buds at much higher medium concentrations. The activity of DHZR is intermediate with respect of these two groups of cytokinins There is no consistent pattern in the difference in activity between a cytokinin base and the corresponding riboside. Z and ZR caused the formation of maximally one flower bud on an explant (not shown).

## Initiation period for flower bud formation.

The question arises whether the cytokinins applied are needed during the entire incubation period. Explants were first incubated on medium with 1$\mu$M of both BA and NAA and then transferred to medium from which BA was omitted. For sake of comparison the transfer experiment was also carried out using media with and without NAA or without both hormones (Fig. 2). From these experiments it became clear that the presence of BA was only required during the first 2 d and NAA during the first 5 d of culture for optimal flower bud formation.

## BA metabolism and flower bud formation.

To investigate which derivatives of BA might be active in flower bud formation, explants were incubated for 4 d on [$^3$H] BA and then transferred to a medium without BA (Fig. 3). The tissues were pulse-labeled at an inductive (1 $\mu$M) and a non-inductive (0.1$\mu$M) BA level. The levels of BA and its metabolites inside the explants were determined as described in Materials

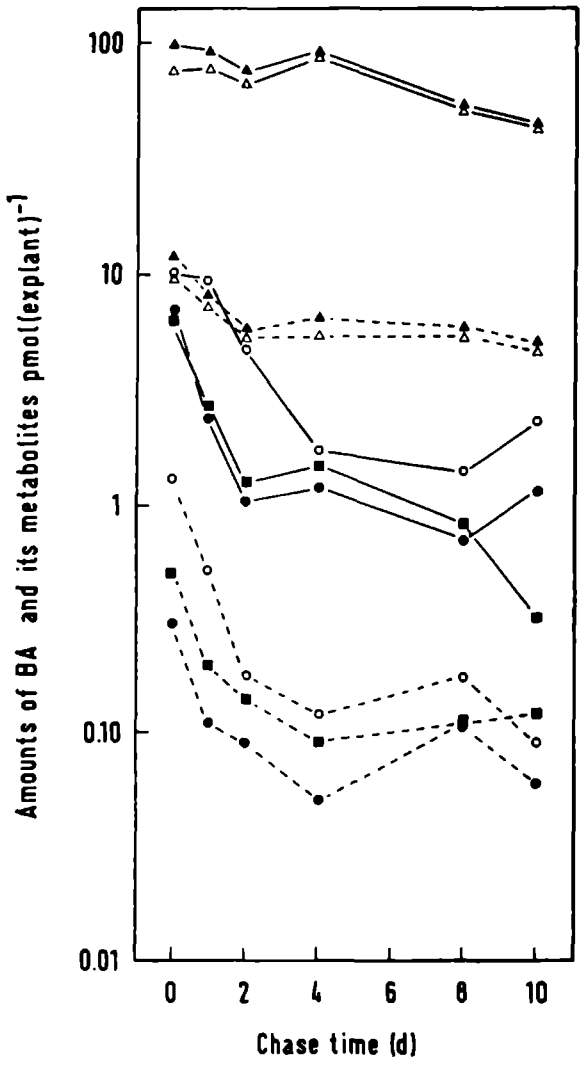


Fig. 3. Metabolism of BA at inductive and non-inductive BA concentrations. Groups of 10 explants were pulse-labeled for 4 d on medium containing 0.1$\mu$M [$^3$H]-BA (broken lines) or 1$\mu$M [$^3$H]-BA (solid lines) and then transferred to medium without BA. ▲, BA plus metabolites, △ 7G-BA; ○ nucleotides, ■ BA; ●, 9R-BA. *Values are means of duplicate experiments*

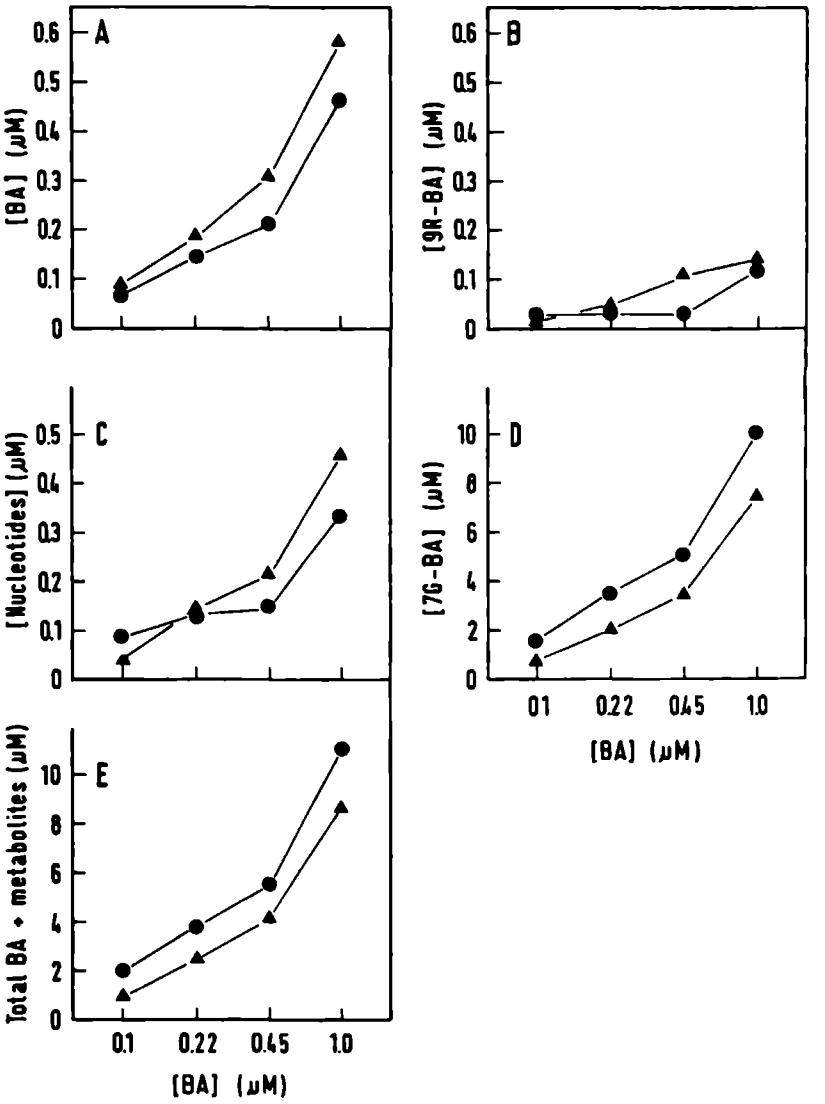


Fig. 4 Uptake and conversion of BA after 24 h of culture on medium containing 1 μM BA (●) or 1 μM BA plus 2 μM IP (▲). *Results are means of duplicate experiments*

and Methods. We found that the equilibrium between BA and the conjugates was independent of the cytokinin concentration in the pulse medium. The uptake of BA was proportional to the medium concentration. As a consequence, there was a constant difference of approximately one decade in the level of BA and its metabolites between explants incubated at the low and at the high BA

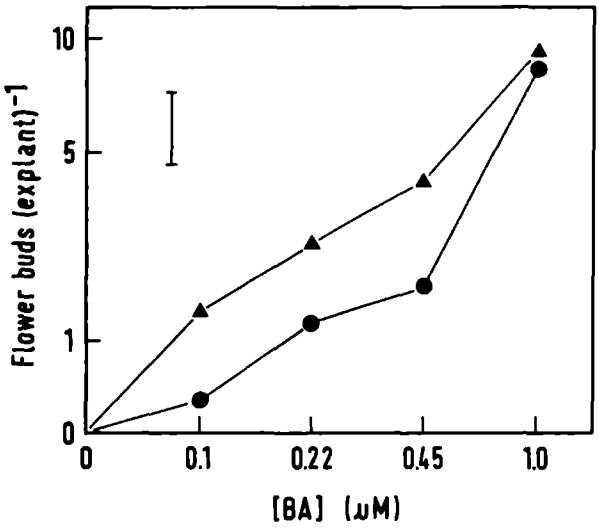


Fig 5. Effect of BA alone ( ● ) and in combination with 2.2μM IP ( ▲ ) on flower bud formation *in vitro*. Vertical bar: LSD at $p = 0.05$

concentration. Immediately after the pulse 7G-BA is the most abundant metabolite (approximately 75 pmol per explant), BA itself, the nucleotides and 9R-BA each form a fraction of about 7 to 10 pmol per explant. With increasing chase time the levels of BA, the nucleotides and 9R-BA decline during the first 4 days of culture to a constant level, the amount of 7G-BA decreases during the whole chase period (to a level of about 50% of the initial amount). The results also show that there is a leaking of BA and its metabolites, especially 7G-BA, out of the explants, either directly, or after interconversion into some other metabolites.

## Synergism of BA and IP.

The absence of a difference in the equilibrium between BA and its metabolites under inductive and non-inductive conditions precludes the identification of physiologically active derivatives. Therefore, we attempted to shift the equilibrium by competitive inhibition of BA conjugation. IP was chosen as a competitor because of its low cytokinin activity (Fig. 1). If IP is acting as a competitive inhibitor of BA conversion, it may cause an increase of the endogenous concentration of the free BA. Such an increase was indeed found (Fig. 4A). In seven out of eight measurements, the BA concentrations in explants incubated on BA plus IP were higher than in the corresponding explants cultured on BA alone. This means that IP

Table 1. Effect of BA on IP metabolism Explants were incubated for 24 h on radiolabeled IP with or without BA Values are means of duplicate experiments (μM)

| Metabolites | Incubation conditions | | |
|---|---|---|---|
| | 2.2 μM IP | 2.2 μM IP + 1 μM BA | 10 μM IP |
| IP | 1.9 | 5 8 | 15.6 |
| Ade | 1.8 | 2 4 | 7.2 |
| IPA | 0.2 | 0 8 | 1.2 |
| Ado | 0.5 | 0 5 | 3.6 |
| 7G–IP | 9.0 | 7.4 | 25.1 |
| Nucleotides | 0.8 | 1.2 | 3.0 |
| Total | 14.5 | 19.4 | 59.8 |

leads to a significant rise in BA concentration ($p < 0.05$). There is a small but insignificant increase in the levels of 9R–BA (Fig. 4B) and the nucleotides (Fig. 4C). The endogenous concentration of BA increased at the expense of the glucoside ($p < 0.01$) (Fig. 4D), and in spite of the reduction of BA uptake ($p < 0.01$) caused by IP (Fig. 4E). At the physiological level the two hormones act synergistically (Fig. 5). The combination of the two cytokinins resulted in a significant increase in bud number at BA concentrations below 1μM IP had no effect on the number of buds at 1 μM BA, presumably because the bud number at this BA concentration is already maximal. The IP concentration used did not lead to flower bud formation when applied alone (Figs. 1 and 5). The increase in bud number upon incubation on BA and 2.2 μM IP is not due to the change in IP metabolism (Table I). Although BA inhibits IP conjugation, the amount of IP and its metabolites in the presence of BA is much lower than after administration of 10 μM IP without BA (Table I). No buds are formed under the latter conditions.

# DISCUSSION

The thin–layer culture system used in this system enabled us to relate cytokinin metabolism to flower bud regeneration. All cytokinins tested induced the same maximal number of flower buds with the exception of Z and ZR which were

virtually ineffective. The similarity in the maximal response to a variety of different cytokinins suggests that these compounds bind to the same receptor BA, 9R-BA and DHZ proved to be the most active cytokinins whereas DHZR and especially IPA and IP were effective at much higher medium concentrations. The high IP and IPA concentrations needed for optimal bud formation are not a result of an extensive breakdown of the molecule by side chain cleavage (Table I). Only a small fraction is lost in this way. The uptake and also the amount of the conjugates formed does not differ very much between the rather inactive IP and the highly active BA. This suggests that differences in sensitivity of the common cytokinin receptor to IP and BA account for the differences in flower bud formation. Our results do not substantiate reports on low activity of natural cytokinins in flower bud regeneration (10, 19, 20). One reason for this discrepancy might be that these earlier studies were carried out at a limited range of cytokinin concentrations which did not include the optimal levels.

Application of BA is only necessary for 2 d to initiate flower bud formation. After withdrawal of the hormone, the level of BA and its metabolites, with the exception of the inactive 7G-BA, remained at a constant level during the outgrowth of the flower buds. This level was proportional to the concentration of the plant growth regulator in the culture medium (Fig 3). This means that regulation of flower bud development is not correlated with a specific type of cytokinin metabolism. The data rather suggest that the role of cytokinin metabolism is to maintain a steady level of BA and some metabolites after the initiation phase The effect of BA on flower bud formation is considerably enhanced by IP at a concentration which is by itself physiologically inactive (Fig 4) In view of the extensive metabolism of BA, (Fig. 5) an explanation for the synergistic action of BA and IP is that IP competitively inhibits BA inactivation. Evidence for such a mechanism comes from the observation that IP causes an increase in the endogenous concentration of BA and a reduction in the level of 7G-BA. This result is not unexpected since the enzymes responsible for cytokinin conversion are most likely not cytokinin-specific, but play a role in the metabolism of purine bases, ribosides and ribotides in general (2, 3, 4, 5). The endogenous concentration of the free cytokinin base is most likely the cause of the increased bud number but any additional physiological activity of 9R-BA and the nucleotides cannot be excluded The fact that application of cytokinin-ribosides to the medium leads to bud formation does not imply that the riboside is active by itself since it can be converted into other

conjugates (data not shown). Letham et al. (14) have suggested that during growth, BA, 9R-BA and nucleotides are active in radish cotyledons. In growing tobacco cell cultures Laloue and Pethe (11) only found activity of the free base.

In our system, the dilemma whether only BA or also some of its metabolites are active is not completely resolved. One other point of interest is the involvement of any endogenously synthesized cytokinins in flower bud formation. This problem is presently under investigation.

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# CHAPTER 4

## Interconversions of benzyladenine metabolites in pedicel explants of tobacco

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Submitted for publication.

# Interconversions of Benzyladenine Metabolites in Pedicel Explants of Tobacco


## ABSTRACT

Uptake and conversion of benzyladenine were studied during culture *in vitro* of pedicel explants of tobacco (*Nicotiana tabacum* L.) Once taken up in the tissue, benzyladenine easily leaked out of into the medium. Less than half of the amount that entered the cells was trapped by conversion into a number of polar metabolites. During the first day of culture benzyladenine–7–glucoside was the predominant metabolite. It was formed either directly from benzyladenine or from benzyladenine–ribotides. Benzyladenine taken up during the second day was conjugated faster than before but formation of the glucoside lagged behind. As a result, the ribonucleotide pool synthesized from the newly–entered benzyladenine was greater than the amount of 7–glucoside. The glucoside was slowly reconverted into ribonucleotides later in culture. Since ribonucleotides are easily hydrolizable to the free base, it was concluded that the glucoside can be considered a slow release source of benzyladenine for the tissue.



Keywords––Cytokinin, BA, BA–metabolism, tissue culture, tobacco.


## INTRODUCTION

The presence of cytokinins and auxins is a prerequisite for culture *in vitro* of many plant tissues. In studying their role in regeneration it is important to know the concentrations of the physiologically active hormone or hormone derivatives within the tissue. Important factors influencing these concentrations are: hormone uptake, conjugation, interconversion of the conjugates, activity of the conjugates, compartmentation and breakdown.

The natural cytokinin zeatin can be metabolized to the mono–, di–, and triribotides (nucleotides), the 7– and 9–glucoside, the O–glucoside, the 9–riboside and the 9–alanin. Zeatin can also be broken down by side chain cleavage (Letham and Palni, 1983). From benzyladenine (BA) the same metabolites are formed with the exception of the O–glucosides (Laloue and Pethe, 1982; Van der Krieken et al.

1988; Letham and Palni, 1983). The nucleotides are considered to be storage forms (Laloue and Pethe, 1982; Horgan, 1983) or to be physiologically active (Van Staden, et al. 1986). The 7-glucoside is assumed to be a storage form (Gawer, Laloue, Terrine, and Guern, 1977; Laloue and Pethe, 1982; Letham et al. 1982) whereas the 9-riboside could be physiologically active (Letham et al. 1982). The cytokinin base is considered to be physiologically active (Van der Krieken et al. 1990, Laloue and Pethe 1982; Letham and Palni, 1982). In bud regenerating pedicel explants of tobacco (Tran Thanh Van 1973) more than 90% of the cytokinin BA was found to be conjugated after 24 h of uptake (Van der Krieken et al., 1988). The most abundant conjugate was the 7-glucoside (7G-BA), whereas the concentrations of the BA-ribotides (nucleotides) and especially the 9-riboside (9R-BA) were much lower. Increasing the BA concentration in the medium led to a change in the composition of the metabolite pool: the proportions of BA, 9R-BA and nucleotides increased at the expense of 7G-BA.

Several questions concerning BA metabolism have not been answered yet. It is not known whether cytokinin metabolism changes with the duration of the incubation of the tissue. Changes during culture in the conjugation of 1-naphthaleneacetic acid which is the auxin used in this system, and interconversions of its conjugates have been reported (Smulders et al. 1989). There are no data on the stability of the cytokinin conjugates during organ regeneration *in vitro*. These problems can be solved by following BA metabolism in the explants for an extended period of time. Pedicel explants are suited for such a study because they have a large surface area in contact with the culture medium which leads to rapid hormone uptake.

In this paper we present data on the time course of BA metabolism in this system. The levels of the metabolites during and after labeling with radioactive BA were followed at various intervals to obtain insight in the constancy of the conjugation process and in the stability of the BA conjugates.

# MATERIALS AND METHODS

## *Culture in vitro*

Flowers of tobacco (*Nicotiana tabacum* L. cv. Samsun) were picked with pedicels attached at anthesis when the inflorescence was in full bloom (stage IV Croes et al. 1985). Small explants (8x1 mm) were cut from the flower stalks and cultured on

Murashige and Skoog (1962) medium with 125 mol $m^{-3}$ glucose and 1% agar, according to Van den Ende et al. (1984). The NAA concentration was 1 $\mu$M throughout. The concentration of BA varied as indicated with each experiment. To study uptake and metabolism, [2,8-$^3$H] BA (specific activity 15.5 TBq $mol^{-1}$, Amersham) or [8-$^{14}$C]-BA (specific activity 2.0 TBq $mol^{-1}$, Amersham) was included in the medium.

## *Uptake of BA in the explants*

The total uptake of BA in the explants was measured after exposure to [$^3$H] BA for various periods of time. Groups of 10 explants were extracted in modified Bieleki's fixative (Laloue and Pethe 1982) overnight and the radioactivity in the extracts was determined by liquid scintillation counting.

## *Separation of BA-metabolites by TLC*

BA and its metabolites were extracted from groups of 10 explants according to Laloue and Pethe (1982). After incubation, the explants were homogenized in 3 $cm^3$ modified Bieleski fixative and the extract was centrifuged. The pellet was washed in 1 $cm^3$ fixative, the pooled supernatants were dried at 40 °C under nitrogen, and the residue was dissolved in 50% ethanol. The extracts were chromatographed according to Tao et al. (1983) on silica gel 60 $PF_{254}$ plates. Merck) with authentic 7G-BA, 9R-BA, 9R-BA monophosphate and BA as standards. The solvent used was *n*-butanol: 14 M $NH_4OH$:water (6:1:2 v/v/v), upper phase. To quantify the radioactivity on the TLC plates, the UV absorbing spots were scraped off and extracted with 0.5 $cm^3$ water for 2 h. After addition of 4 $cm^3$ Lumagel (Lumac), radioactivity was measured by liquid scintillation counting.

## *Release of BA and its metabolites into the culture medium*

After incubation of 10 explants on MS medium containing [$^3$H]-BA for 1 h, the explants were washed and transferred to a 3-mm thick agar block with a surface area of 1 $cm^2$. Twenty-four hours after transfer they were removed and the agar block was eluted two times with 1 $cm^3$ of distilled water for 24 h. The water phases were evaporated under nitrogen at 40 °C, the residue was dissolved in 50 % ethanol, and the BA metabolites were separated by thin-layer chromatography (TLC) as described below.

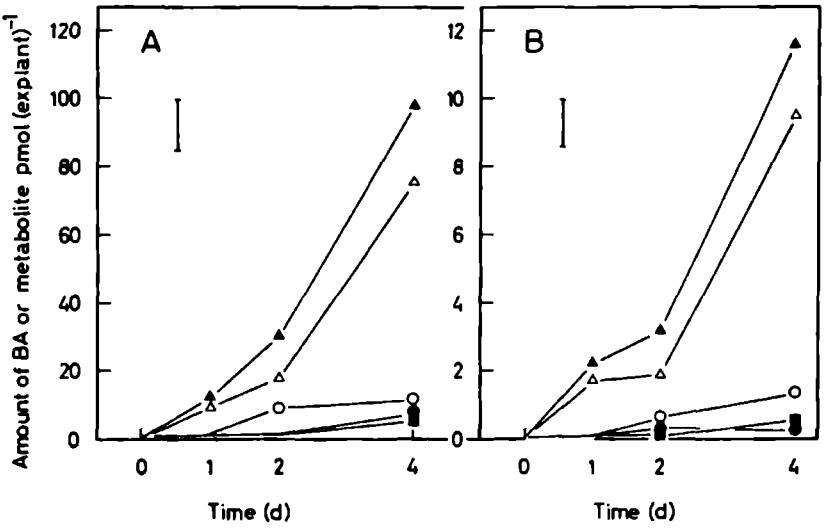


FIG. 1. Uptake and metabolism of BA during continuous incubation. Explants were incubated on Murashige and Skoog medium with 1 mmol m$^{-3}$ (A) or 0.1 mmol m$^{-3}$ BA (B) and 100 kBq cm$^{-3}$ [3H]-BA. ▲ uptake, △ 7G-BA, ○ nucleotides, ■ BA, ● 9R-BA. 20 explants were used per treatment. Vertical bar: least significant difference at $p = 0.05$.

# RESULTS

## *Uptake and metabolism of BA during continuous incubation*

The uptake and metabolism of BA were determined during a 4-day incubation at medium concentrations of 0.1 mmol m$^{-3}$ and 1 mmol m$^{-3}$ (Fig. 1). During the second half of the four-day incubation period the uptake rate was higher than during the the first half. Throughout incubation the percentage of the conjugates was approximately 93%. The relative abundance of the conjugates, however, changed. With increasing incubation time the proportion of nucleotides increased while that of 7G-BA decreased. The medium concentration of BA did not influence the relative amounts of the various compounds.

## *Changes in metabolism*

We studied the observed change in BA uptake and metabolism in more detail by comparing BA uptake and conversion during the first and second day of culture. The uptake was higher during the second day of culture at both 0.1 and 1 mmol $m^{-3}$ BA (Table 1). The conjugation during the second day of culture differed from that during the first day (Fig. 2). After 1 d 7G–BA was the most abundant metabolite whereas during the second day more nucleotides were formed than 7G–BA. The relative levels of 9R–BA and BA were approximately 7% throughout. The presence of BA during the first day of culture slightly increased the uptake during the second day at a medium concentration of 1 mmol $m^{-3}$ (Table 1), but did not influence the proportions of the various conjugates formed.

TABLE 1. *Uptake of BA in 24 h during the first and second day of culture.* Explants were either immediately exposed to 1 $\mu$M [$^3$H] BA (I) or preincubated for one day on medium with (II) or without (III) 1 $\mu$M BA

| BA concentration in medium (mmol $m^{-3}$) | BA uptake (pmol explant$^{-1}$) | | |
|---|---|---|---|
| | I | II | III |
| 0.1 | 2.1 | 3.3 | 2.9 |
| 1 | 12.2 | 19.6 | 16.9 |

## *Stability of the BA metabolites*

The fate of the BA–metabolites once formed was examined in an experiment in which BA taken up by freshly cut explants during a pulse of 1 h on 10 mmol $m^{-3}$ [$^{14}$C]–BA was chased on medium with or without BA (Fig. 3). The distribution of label in BA and its metabolites was followed during a three days. The total amount of BA plus its metabolites decreased by 70% during the first 9 h of the chase period and remained stable afterwards. Immediately after the pulse, the free base was the most abundant compound (approximately 45%). With increasing chase time the BA level dropped to approximately 2% after 24 h. From 9 to 24 h the level of 7G–BA increased, whereas all another compounds decreased. The nucleotide level increased slowly but reproducibly upon prolonged incubation. The level of 9R–BA is initially low and beyond detection after 24 h. The course of BA metabolism did not depend on the BA concentration in the chase medium. NAA had no effect on BA metabolism.

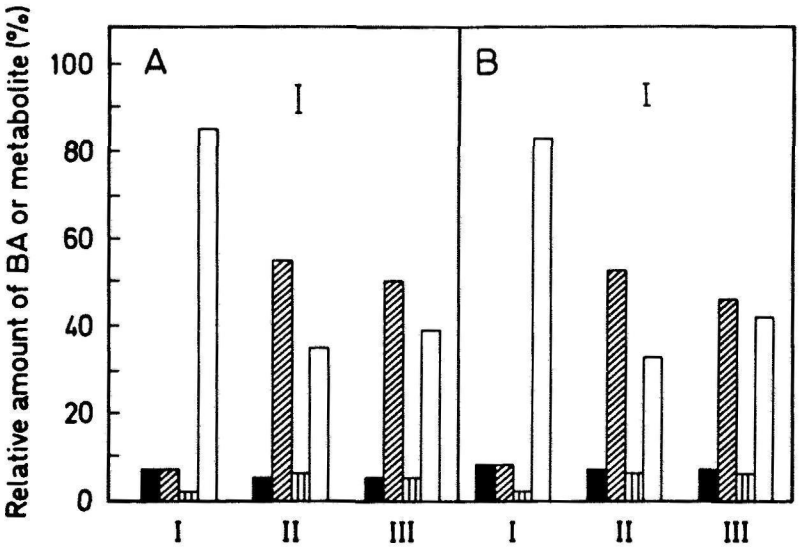


FIG. 2. Relative amounts of BA and its metabolites after incubation for 1 d on 1 mmol $m^{-3}$ (A) or 0.1 mmol $m^{-3}$ (B), and 100 kBq $cm^{-3}$ [$^3$H]-BA. I, no preincubation; II, 1 d preincubation on medium with unlabeled BA, 1 $\mu$M (A) or 0.1 $\mu$M (B); III, 1 d preincubation on medium without BA. Black, BA; oblique stripes, nucleotides; vertical stripes, 9R-BA; white, 7G-BA. Vertical bar: least significant difference at $p = 0.05$.

A similar pulse-chase experiment was carried out on the second day of culture. Explants were preincubated for one day on standard medium and then pulse-labeled for 1 h at 1 mmol $m^{-3}$ [$^3$H]-BA. The chase was on medium with or without 1 mmol $m^{-3}$ BA (Fig. 4). Like in the previous experiment, the total amount of label dropped within 10 h to less than half the amount present immediately after the pulse. The amount of BA declined even faster than in not-preincubated tissues. The minimal levels of the nucleotides, 9R-BA and BA were reached in 5.5 to 9 h of chase as compared to 24 h when the explants had not been not preincubated (Fig. 3). The drop in the nucleotide level which coincided with an increase in 7G-BA is more pronounced than in the previous experiment. The results suggest that preincubation resulted in an acceleration of BA conversion. Explants labeled on the second day contained relatively more nucleotides and less 7G-BA. The change in BA metabolism did not depend on the presence of BA in the chase medium.

## *Leakage of BA and its metabolites*

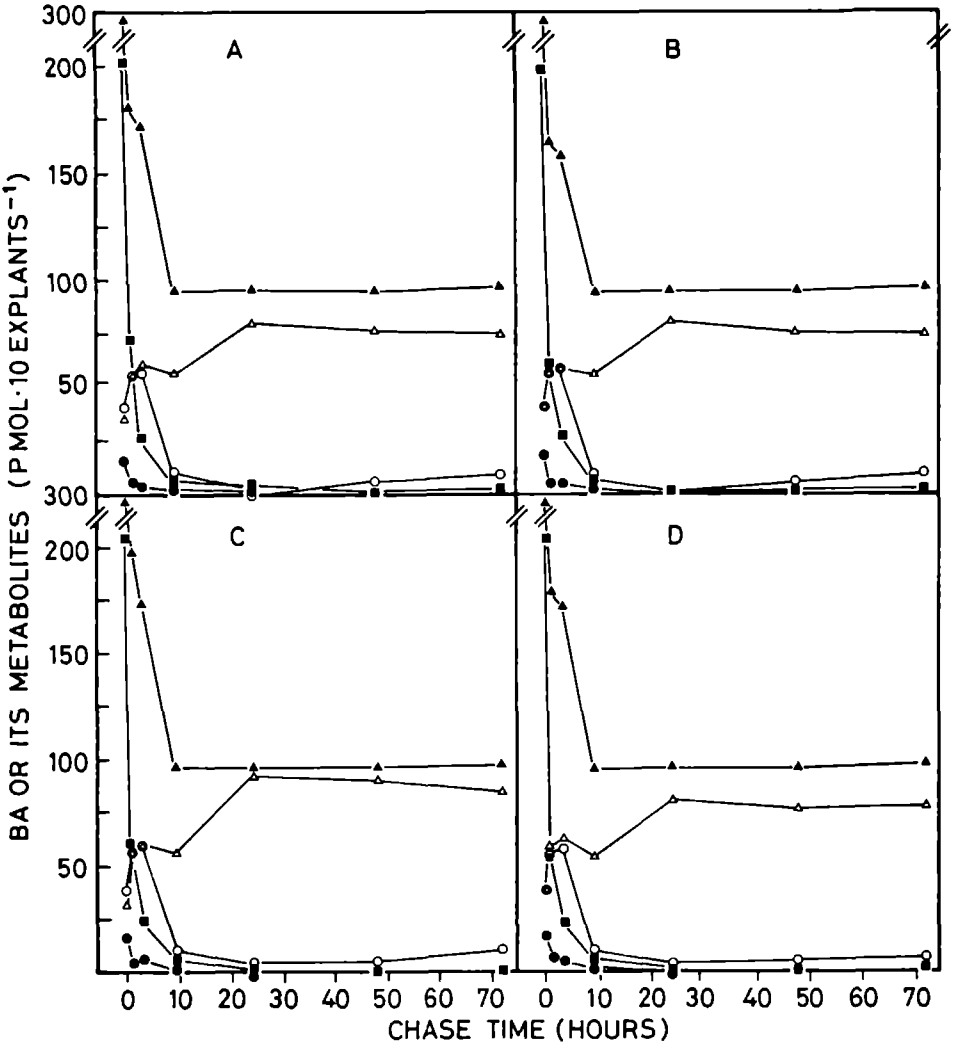


FIG. 3. Metabolism of BA during early flower bud formation. After a 1 h pulse of 20 explants on medium with 10 $\mu$M (100 kBq cm$^{-3}$) [$^{14}$C]-BA and 1 $\mu$M NAA, the BA and metabolite composition was determined after different chase times on media with 1 $\mu$M NAA in combination with 1 $\mu$M (A) or 0.1 M BA (B) or 0 1 $\mu$M NAA in combination with 1 $\mu$M (C) or 0 1 (D) $\mu$M BA. ▲, uptake, △, 7G-BA; ○, nucleotides, ■, BA, ●, 9R-BA.

The loss of label observed in the pulse–chase experiments (Figs 3 and 4) and the even faster reduction in the amount of BA itself might be caused by leakage of BA from the cells into the medium. This possibility was studied by transferring explants after a 24 h incubation to water solidified with 1% agar. After 24 h and 48 h, the medium under the explants was extracted and the radioactivity in the extracts was

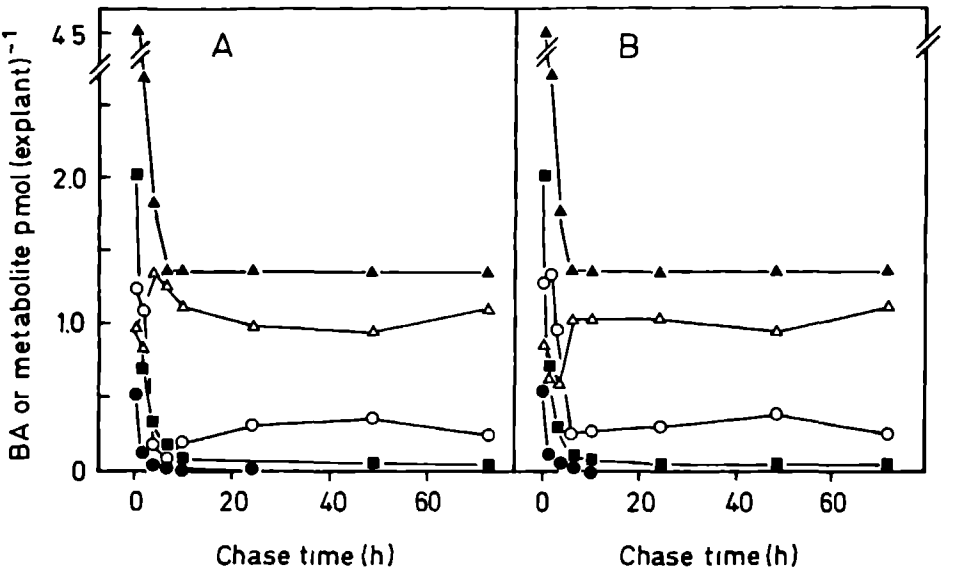


FIG 4 Metabolism of BA after a 1 d preincubation After preincubation on 1 $\mu$M BA and 1 $\mu$M NAA, 20 explants were pulsed for 1 h on medium with 1 $\mu$M [$^3$H]-BA, $10^6$ kBq cm$^{-3}$. The BA-metabolites were quantified after different chase times on media with 1 $\mu$M (A) or 0 1 $\mu$M BA (B) ▲, uptake; △, 7G-BA, ○, nucleotides, ■, BA, ●, 9R-BA

analyzed. Twenty percent of the radioactivity had leaked out the explants. Most of it was recovered as free base (90%) and only 5% was in 7G-BA (Table 2).

## *DISCUSSION*

The culturing of pedicel explants offers the possibility to study the uptake and conjugation of cytokinins, and interconversion of cytokinin metabolites during culture *in vitro*. Upon prolonged incubation the rates of BA uptake and conjugation increased. The principal metabolites, the BA-ribotides and 7G-BA, did not accumulate in the same proportions throughout culture. The nucleotides and 7G-BA were probably interconverted during culture.

The metabolism of BA taken up in the tissues proceeded in three different phases. In the first period of approximately nine hours, the major part of BA that was taken up during a the hour preceding the measuring period leaked back into the culture medium (Fig. 3, Table 2) Over 90 percent of the BA that did not leak out of the explants was trapped into conjugates. In the second phase, lasting from from 9 to 24 hours, the level of 7G-BA rose to a maximum at the expense of all other conjugates. During the third phase, which lasted at least for a fourty more

TABLE 2. BA leakage to the medium. A group of 30 explants was incubated for 24 h on medium with 1 mmol m-3 [3H]-BA and then placed on a block of water-agar for 48 h. After 24 h the agar block was refreshed. After extraction of the agar, the metabolites were separated by thin layer chromatography and the radioactivity in each of them was counted.

| Compounds | Radioactivity leaked out the explants (%) | |
|---|---|---|
| | 24 h | 48 h |
| BA | 92 | 88 |
| 9R-BA | 3 | 7 |
| 7G-BA | 5 | 5 |
| Nucleotides | 0 | 0 |

hours, a small but reproducible decrease of the 7G-BA level coincided with an increase in the nucleotide level. Since nucleotides and 7G-BA cannot be directly interconverted (Letham and Palni 1983), the followed pathway must include hydrolysis of 7G-BA and ribosylation of the free base followed by phosphorylation. Interconversion has also been observed for the 7-glucoside and the nucleotides of dihydrozeatin (Van der Krieken unpublished). During the second day of culture both BA uptake and conjugation were speeded up (Fig. 4) and there was a change in the proportions of newly-formed nucleotides relative to 7G-BA. This suggests that during the second day the increase in conjugation rate was mainly due to a rise of nucleotide formation, and that glucoside formation did not keep up with the enhanced BA influx.

The medium concentrations of BA and also NAA (unpublished data) did not have an effect on cytokinin conversion. In a much wider range of concentrations, flooding of the tissues with high BA levels has been found to reduce the percentage of the conjugated forms (Van der Krieken et al. 1988). This is presumably due to saturation of the conjugating enzymes. A limited effect of BA on its own metabolism has also been observed before in radish-cotyledons and tobacco (Letham et al., 1982).

The BA glucoside may be considered an inactive storage form of BA because it can slowly be hydrolyzed to BA. Our results suggest that the glucoside is

converted into nucleotides in the later stages of culture. The physiological significance of this process might be that cytokinins are available in a form that cannot leak out of the tissue (Table 2) and can easily be converted into active cytokinin. During culturing active BA could be provided by the nucleotide pool in this way. A similar role for the cytokinin nucleotides in tobacco cell cultures has been proposed (Horgan 1987).

In conclusion, the measurements of the levels of the hormone and the metabolites during incubation have made clear that BA conjugation changes during culture, and have demonstrated that the conjugates are in a dynamic equilibrium due to continuous interconversions.

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# CHAPTER 5

## Dose-dependent induction of flower bud formation in vitro by dihydrozeatin

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Submitted for publication.

# Dose–dependent induction of flower bud formation *in vitro* by dihydrozeatin


## ABSTRACT

Flower stalk explants of tobacco cultured on a medium with an auxin and a cytokinin regenerate flower buds within fourteen days. The optimum medium concentrations of dihydrozeatin and benzyladenine are both one $\mu$M. However, the presence of dihydrozeatin in the culture medium is necessary for minimally seven days (initiation period), whereas BA is only needed during the first four days. This differences in the length of the initiation period is neither due to a lower uptake rate of DHZ nor to differences in conjugation of the two cytokinins into inactive metabolites, or differences in breakdown or compartmentation. Inside the tissue, the DHZ concentration optimal for bud formation is two to three times the optimal concentration of BA. This suggests that the affinity of the cytokinin receptor is greater for BA than for DHZ. At a medium concentration of 1 $\mu$M, DHZ is taken up at least twice as fast as BA. Therefore, the dose of DHZ accumulated during maximal bud formation is much higher than the corresponding of BA. It is concluded from the combined data that DHZ is less active in inducing flower bud formation than BA.



Key words– Cytokinin, cytokinin–metabolism, dihydrozeatin, benzyladenine, tissue culture, tobacco.


Abbreviations: Ade, adenine; Ado, adenosine; BA, N6–benzyladenine; DHZ, dihydrozeatin; 7G–DHZ, dihydrozeatin–7–glucoside; LSD, least significant difference; NAA, 1–naphthaleneacetic acid; (OG)–DHZ, O–glucoside of dihydrozeatin; 9R–DHZ, DL–dihydrozeatin–riboside; 9R(P)–DHZ, DL–dihydrozeatin–riboside monophosphate; TLC, thin–layer chromatography.

## Introduction

The presence in the medium of cytokinins and auxins is a prerequisite for culture of many plant tissues *in vitro*. The role of these hormones in organ regeneration is commonly studied by relating the hormone concentration in the medium to the magnitude of the response (Skoog and Miller 1957, Tran Thanh Van 1981). For a

detailed analysis of the relation between hormone concentration and response, it is important to determine the concentrations of the physiologically active hormone or hormone derivatives within the tissue. Cytokinins are converted into a number of derivatives (Letham and Palni 1983, Horgan 1987), but most likely only the free base is active (Van der Krieken et al. 1990, Laloue and Pethe 1982)

In thin-layer explants of tobacco pedicels the various cytokinins show different activities in promoting bud formation. BA and DHZ induce maximal bud formation at the same medium concentration, but IP is 20 to 40 times less active and Z possesses almost no activity (Van der Krieken et al. 1990). That DHZ and BA induce the maximum number of flower buds at the same medium concentration in explants continuously exposed to the hormone, does not necessarily mean that both cytokinins are equally active. If the cytokinin need not be present during the entire culture period, the minimum exposure times for BA and DHZ required for maximal bud formation might be different. BA was found to initiate maximal flower bud formation in the first 4 days of culture (Van den Ende et al. 1984). This could mean that the physiological response is dependent on the dose of the hormone accumulated in the tissue. A dose-dependent response of the same tissue culture system to auxin has recently been found (Smulders et al. 1990). If the initiation period for DHZ is longer or shorter, the difference could be due to differences between DHZ and BA in uptake rate, in conjugation and in biological activity. To investigate these possibilities the lengths of the incubation period for DHZ and BA and the doses of BA and DHZ accumulated during this period should be compared.

In this study the activities of DHZ and BA were compared by measurement of the initiation periods for maximal flower bud formation and of uptake and conjugation of these cytokinins.

# MATERIALS AND METHODS

## *Culture in vitro*

Flowers of tobacco (*Nicotiana tabacum* L. cv. Samsun) were picked with pedicels attached at anthesis when the inflorescence was in full bloom (stage IV, Croes et al. 1985). Small explants (8x1 mm) were cut from the flower stalks and cultured on Murashige and Skoog (1962) medium with 125 mM glucose and 1% agar (Difco Bacto), according to Van den Ende et al. (1984). 1-Naphthaleneacetic acid (NAA)

was added at a medium concentration of 1 $\mu$M, DHZ and BA concentrations were added as indicated for each experiment. Flower buds were counted after 14 days of culture. The data were entered in the figures after the logarithmic transformation X = ln (bud number + 2). The LSD was calculated from the transformed values and presented in the figures for comparison of treatment means. The explants were weighed prior to extraction of DHZ and its metabolites. To study uptake and metabolism, [2, 3(n)-$^3$H] DHZ (specific activity 1520 TBq mol$^{-1}$ Amersham) or [2,8-$^3$H] BA (specific activity 155 TBq mol$^{-1}$, Amersham) was included.

## *Separation of DHZ-metabolites by TLC*

DHZ and its metabolites were extracted from groups of 10 explants according to Laloue and Pethe (1982). After incubation, the explants were homogenized in 3 ml modified Bieleski fixative and the extract was centrifuged. The pellet was washed in 1 ml fixative, the pooled supernatants were dried at 40 °C under nitrogen, and the residue was dissolved in 50% ethanol. The extracts were chromatographed according to Tao et al. (1983) on silica gel 60 $PF_{254}$ plates Merck) with authentic 7G-DHZ, 9G-DHZ, (OG)-DHZ, 9R-DHZ, 9R(P)DHZ (nucleotides), DHZ, Ado and Ade, as standards (purchased from Apex Organics). In the first dimension, the plates were developed twice with *n*-butanol:14 M $NH_4OH$:water (6:1:2 v/v/v, upper phase) as the solvent. The solvent in the second dimension was *n*-butanol:acetic acid:water (12:3:5, v/v/v). To quantify the radioactivity on the TLC plates, the UV absorbing spots were scraped off and extracted with 0.5 ml water for 2 h. After addition of 4 ml Lumagel (Lumac), radioactivity was measured by liquid scintillation counting. In some experiments separation by one-dimensional TLC proved to be sufficient.

Table 1. Metabolites formed from DHZ after 24 h incubation on MS medium with 1 $\mu$M NAA and 2.2 $\mu$M DHZ. The extract of 10 explants was co-chromatographed on two-dimensional TLC with standards of DHZ metabolites After visualizing the standards under UV-light the spots were scraped off and radioactivity was determined

| Compound | Radioactivity, % |
|---|---|
| 7G-DHZ | 59.3 |
| nucleotides | 12.6 |
| 9R-DHZ | 11.3 |
| DHZ | 9.3 |
| 9G-DHZ | 3.1 |
| (OG)-DHZ | 1.1 |
| Ade | 0.0 |
| Ado | 0.0 |

# RESULTS

## *Flower bud induction by DHZ and BA*

The same number of flower buds was formed after 14 days of culture on medium with 1 $\mu$M of either BA or DHZ (Van der Krieken et al. 1990). The period that DHZ and BA were necessary in the medium to lead to maximal bud formation, the induction period, was determined (Fig. 1) by transferring explants after different culture times on inductive medium to medium without cytokinin. The most striking difference between both cytokinins was observed when they are applied for 2, 3 or 4 days. After a pulse of 3 days, for example, DHZ had hardly any effect, whereas BA showed already 70% of the maximal effect

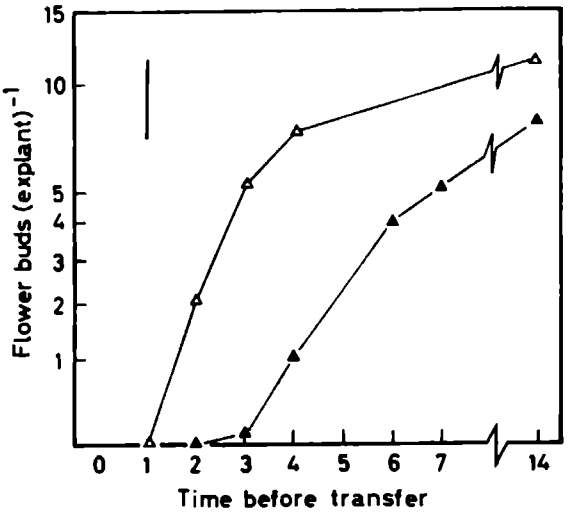


Fig 1 Period during which the presence of DHZ and BA is necessary to induce bud formation Explants were first cultured on complete medium. At the times indicated, they were shifted to media without DHZ (▲), or without BA (△) Vertical bar LSD at $p = 0.05$.

## *DHZ uptake and conjugation*

The uptake rate of DHZ at all medium concentrations tested is larger than the corresponding BA uptake (Fig. 2) ($p < 0.05$). The metabolites formed from DHZ were examined by co-chromatographing a labeled extract of the explants with a mixture of metabolite-standards (Table 1). Radioactive spots were found at the positions of 7G-DHZ, 9R-DHZ, nucleotides, and DHZ itself. Only few counts co-chromatographed with the O-glucoside of DHZR (1%) and 9G-DHZ (3%). No radioactivity was found in the Ade and Ado spots indicating that no side-chain cleavage had occurred. In the following experiments only the four most abundant metabolites will be quantified by one-dimensional TLC.

## *Accumulation and metabolism of DHZ during flower bud initiation*

The uptake and metabolism of DHZ was determined during the initiation period for bud formation (Fig. 3). The total amount of DHZ accumulated increased almost linearly during the 7-day period (Fig. 3A). Only a small fraction is recovered in DHZ which indicates that conjugation is a very fast process. The concentrations of DHZ and the various conjugates were estimated by expressing the accumulated amounts on a fresh weight basis (Fig. 3B). The concentrations of most compounds peak at 3 days after the

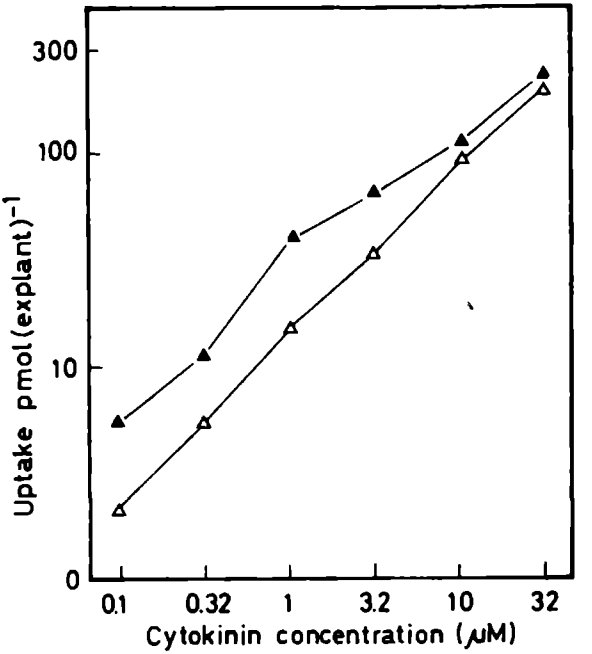


Fig 2 Uptake of DHZ (▲) and BA (△) after 24 h of culture in relation to the DHZ and BA concentration in the medium.

Table 2 Changes in DHZ uptake and metabolism during culture The uptake and metabolism were established in explants cultured for 24 h on medium with 2.2 μM [$^3$H]-DHZ ($10^3$ kBq) after preincubations of different times on the same medium without the radiolabeled DHZ. Values are expressed in pmol explant$^{-1}$ and are means of duplicate experiments

| DHZ and conjugates | Preincubation time (d) | | |
|---|---|---|---|
| | 0 | 3 | 7 |
| DHZ | 6.8 | 13 | 19 |
| 7G-DHZ | 27.1 | 82 | 131 |
| Nucleotides | 13.5 | 220 | 560 |
| 9R-DHZ | 6.6 | 70 | 170 |
| Uptake | 54 | 385 | 930 |

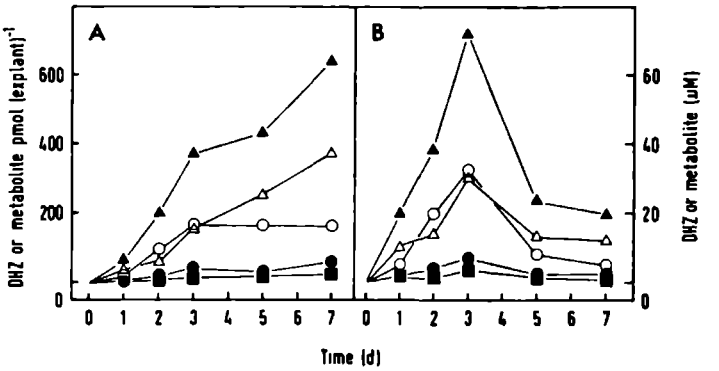


Fig. 3. Uptake and metabolism of DHZ during continuous incubation. Explants were incubated on Murashige and Skoog medium with 2 2μM DHZ and 100 kBq ml$^{-1}$ [3H]-DHZ ▲ uptake; △ 7G-DHZ; ○ nucleotides, ■ DHZ, ● 9R-DHZ. 20 explants were used per treatment.

start of incubation. From a comparison between Figs. 3A and B, it can be deduced that the increase in fresh weight of the explants is responsible for the decline in most concentrations after 3 days. The concentration of DHZ itself remained at a level of 1– 2 μM throughout the 7 days of culture.

### *Changes in DHZ uptake and metabolism during culture*

Previous experiments had revealed that the accumulated dose of BA is the result of BA uptake and leakage back into the medium (Van der Krieken et al. 1990). To check for release of DHZ from the explants, we cultured explants on inductive medium for various periods of time prior to a 24–h incubation on radiolabeled DHZ. Indications for an extensive leakage were found. The amount of DHZ taken up during the fourth day (Table 2) is much greater than the difference in DHZ content between the third and fifth day (Fig. 3A).

The proportion in which the various metabolites were formed changed during culture time. Whereas at day 1 and day 4 more nucleotide than 9R–DHZ was synthesized, the reverse was found at day 8. As a result the relative abundances of these compounds in the conjugate pool gradually changed (Fig. 3A).

## DISCUSSION

Culturing of superficial tissue of flower stalks of tobacco showed that DHZ and BA are not equally active in inducing flower bud formation *in vitro*. The initiation period

for bud formation on DHZ is almost two times longer than on BA (Fig. 1). This difference is not caused by slower uptake, greater conjugation into inactive conjugates or more extensive breakdown of DHZ relative to BA.

The total amount of DHZ taken up and the resulting DHZ concentration in the explants are dependent on a number of interrelated factors. The linear relation between uptake rate and medium concentration (Fig. 2) suggests that at least at the higher concentrations a considerable portion of the hormone, once taken up, is trapped inside the explants. Previous studies on BA metabolism have shown that the equilibrium between BA and its conjugates does not vary with the uptake rate (Van der Krieken et al. 1988) and that the conjugates, in contrast to BA itself, cannot easily leave the cell (Van der Krieken et al. 1990). Since the chemical polarities of DHZ and BA are almost equal, this may also hold for DHZ.

After approximately three days, the explants started growing and, therefore, become bigger sinks. That sink size affects the uptake rate of DHZ is clear from the observation that on day 8 much more DHZ entered the tissue than on day 4 (Table 2). However, the enhanced uptake rate did not lead to an increase in the accumulation rate (Fig. 3A). This means that for a considerable part uptake of DHZ is balanced by leakage from the tissue into the medium.

At a medium concentration optimal for bud formation the DHZ level inside the explants was 1 – 2 $\mu$M throughout the initiation period (Fig. 3B), which is two to three times the optimal concentration of BA (Van der Krieken et al. unpublished). The fact that both cytokinin bases can easily leak out of the tissue shows that they can pass rapidly through the cell membranes. This makes it very unlikely that differences in compartmentation between BA and DHZ would account for the need for different levels of the two cytokinins to achieve maximal bud formation. The most likely explanation for the difference in optimal cytokinin concentration is that the cytokinin receptor has a greater affinity for BA than for DHZ.

The existence of an initiation period after which the presence of cytokinins in the medium is no longer required, points to a dose–dependency of flower bud formation on cytokinin. The physiological meaning of an accumulated dose may be that a cytokinin source from which the active cytokinin can be released is present in the tissue during the period after removal of the hormone from the medium. The unequal lengths of the initiation periods for DHZ and BA make it clear that the optimal dose of DHZ is higher than that of BA. This correlates with the observed difference in tissue sensitivity to the two cytokinins. Since the inducing

concentration of DHZ is higher than of BA, accumulation of a larger dose might be required to maintain a sufficient DHZ concentration for a prolonged period of time. The accumulation of a DHZ dose which exceeds that of BA could take more time.

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# PART 2: THE ROLE OF ENDOGENOUSLY SYNTHESIZED CYTOKININS ON FLOWER BUD FORMATION IN VITRO

# CHAPTER 6

## Endogenous cytokinins in flower bud forming explants of tobacco

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Submitted for publication.

# Endogenous cytokinins in flower bud forming explants of tobacco


## ABSTRACT

The accumulation of the endogenous cytokinins was studied in pedicel explants of tobacco (*Nicotiana tabacum* L.) during regeneration of flower buds *in vitro*. Maximal bud formation was induced by incubating explants on medium containing 1 $\mu$M of benzyladenine or dihydrozeatin. No buds were formed in the absence of cytokinin. The dihydrozeatin levels in explants cultured in the presence or absence of cytokinin were compared by means of a competitive ELISA technique. In explants incubated without a cytokinin, the endogenous dihydrozeatin concentration increased during the first day of incubation and decreased during the second day. However, in explants incubated on medium with benzyladenine no such temporary increase was observed.

The concentration of dihydrozeatin maintained in bud-forming explants cultured in the presence of 1 $\mu$M benzyladenine was only 10 to 15% of the inductive dihydrozeatin concentration in explants cultured on 1 $\mu$M DHZ. This suggests that the endogenous cytokinins only play a minor role in the regeneration of flower buds *in vitro*.


[1] Abbreviations: BA, 6-benzyladenine; DHZ, dihydrozeatin; DHZR, DL-dihydrozeatin-riboside; ELISA, enzyme-linked immunosorbent assay; IP, 6-(-2-isopentenyl)adenine; LSD, least significant difference; NAA, 1-naphthaleneacetic acid; 9R-BA, benzyladenosine; Z, zeatin; ZR, zeatin-riboside.

## INTRODUCTION

The culture of organs on small explants of plant tissue offers the possibility to study differentiation in an environment that is spatially isolated from the whole plant. Differentiation in such explants is regulated in its qualitative and quantitative aspects by the auxins and cytokinins included in the culture medium. Depending on the concentrations of these plant growth regulators roots or shoots (Skoog and Miller 1957), or flower buds (Tran Thanh Van 1981) are induced. For an understanding of the mechanism of action of these plant growth regulators, however, it is necessary to correlate their concentration inside the tissue with the

number of regenerated organs.

Endogenous cytokinin levels have mainly been studied during organ development in whole plants. Endogenous cytokinins were postulated to play an important role in dormancy in potato tubers (Turnbull and Hanke 1985), floral induction (Lejeune et al. 1988), adventitious root formation (Bolmark et al. 1988) and flower bud blasting (Vonk et al. 1986). The role of these cytokinins was inferred from their relative abundance in different plant organs, or different developmental stages of the organs.

Little research has been performed on endogenous cytokinins involved in regeneration processes during tissue culture. Exission of explants has been described to lead to an increase in the endogenous cytokinin concentration (Crane and Ross 1986, Giridhar and Thiman 1985, Mitchel and Van Staden 1983). The rise in the hormone concentration might play a role in early steps of the regeneration processes. For a study of this problem the culture of flower buds on pedicel explants of tobacco is suited because the induction of bud regeneration is depending on cytokinin (Tran Thanh Van 1973, Van den Ende et al. 1984). Uptake and metabolism of exogenously supplied cytokinins in this system have been described and the internal cytokinin concentration resulting from these processes has been established (Van der Krieken et al. 1988, 1990).

In the present study two endogenous cytokinins DHZ and Z and their ribosides were quantified during early flower bud formation by means of a competitive ELISA technique. The levels of endogenous DHZ and Z under inductive and non-inductive conditions were measured and their contribution to the total cytokinin pool in the tissue was calculated. The results have been correlated with the differentiation response of the tissue.

# MATERIALS AND METHODS

## *Culture in vitro*

Flowers of tobacco (*Nicotiana tabacum* L. cv. Samsun) with pedicels attached were picked at anthesis when the inflorescence was in full bloom (stage IV, Croes et al. 1985). Flower buds were regenerated on small explants (8x1 mm) cut from the flower stalks and cultured on Murashige and Skoog (1962) medium with 125 mM glucose and 1% agar (Van den Ende et al. 1984). 1-Naphthaleneacetic acid (NAA) was used at a concentration of 1$\mu$M and BA was added at the inductive

concentration of 1$\mu$M or omitted. Flower buds were scored after 14 d of culture at 25 °C in continuous light. To study uptake and metabolism, [2,8-$^3$H] BA (specific activity 15.5 TBq mol$^{-1}$, Amersham) was included in the medium.

## *Purification of samples for ELISA*

Cytokinin bases and ribosides were extracted from approximately 0.7 g of explants in 8 ml modified Bieleski fixative (Laloue and Pethe 1982). At the beginning of the extraction $10^6$ dpm of [$^3$H]BA and [$^3$H]9R-BA (Amersham, specific activities 15.5 TBq mol$^{-1}$ and 20 TBq mol$^{-1}$ respectively) were added for determination of the recovery of the cytokinins. After homogenization of the explants the sample was centrifuged (15 min, 6000 x g) and the supernatant was dried under nitrogen at 40 °C. The residues were dissolved in 0.5 ml 50 mM HAc, pH 3 and purified on a 1 ml Sep Pak $C_{18}$ cartridge (Waters). Before use the cartridge was rinsed with 50 mM HAc pH 3, 100% methanol, and again 50 mM HAc pH 3. After the extract was brought on the cartridge, the column was rinsed with 15 ml 50 mM HAc pH 3, and the cytokinins were eluted afterwards with 20 ml 35% methanol in 50 mM HAc pH 3. The eluent was dried in a rotating evaporator under vacuum at 40 °C. The residue was dissolved in 1.5 ml 75% ethanol and centrifugated at 10000 x g for 10 min. The volume of the supernatant was reduced to 50$\mu$l under nitrogen at 40 °C. For further purification the extracts were chromatographed according to Tao et al. (1983) on silica gel C60 $PF_{254}$ plates (Merck). Authentic BA, 9R-BA, Z, ZR, DHZ and DHZR were cochromatographed in separate lanes. The solvent used was *n*-butanol: 14 M $NH_4OH$: water (6:1:2 v/v/v), upper phase. After development, the standards were marked under UV-light. The samples with $R_f$ values similar to the standards were scraped off and the cytokinins were extracted with 4 ml 50% ethanol. After evaporation to dryness the extracts were ready for ELISA. For recovery measurements the radioactivity in BA and 9R-BA was determined. The silica was scraped off and extracted with 0.5 ml water for 2 h. After addition of 4 ml Lumagel (Lumac), radioactivity was measured by liquid scintillation counting.

## *Enzyme-linked immunosorbent assay*

An indirect competitive ELISA was used for quantifying DHZ, Z and their ribosides according to Vonk et al. (1986). Dynatech microtiter plates with high protein binding capacity were used. The polyclonal antibodies against ZR and against

DHZR were a generous gift of Mr CR Vonk. The ELISA tests were carried out at least in triplicate, with different dilutions of the samples (Weiler 1984). The data were evaluated by analysis of variance and least significant differences were calculated to compare sample means.

### *Uptake and metabolism of BA and DHZ*

The concentrations of the free cytokinin base resulting from DHZ and BA uptake from the medium were determined according to Van der Krieken et al. (1988).

Table 1 Cytokinin separation on silica gel C60 TLC plates After pre-purification on Sep Pak $C_{18}$ cartridges, the tissue extracts containing the endogenous cytokinins were separated on TLC.

| Cytokinin | $R_f$ value |
|---|---|
| BA | 0.75 |
| IP | 0.75 |
| DHZ | 0.60 |
| Z | 0.60 |
| 9R-BA | 0.44 |
| IPA | 0.44 |
| DHZR | 0.32 |
| ZR | 0.32 |

# RESULTS

### *ELISA assay for DHZ, Z and their ribosides*

The TLC system used for the extraction of the endogenous cytokinins allowed separation of BA and 9R-BA, DHZ and Z however migrated to the same position

Table 2. Cross-reactivity of various cytokinins to the DHZR and ZR-antibodies used in the ELISA test Reactivities are expressed as percentages of the response of DHZR and ZR respectively.

| Cytokinin | DHZR-antibody | ZR-antibody |
|---|---|---|
| BA | 0.05 | 0.03 |
| DHZ | 20.5 | 0.40 |
| Z | 0.10 | 40.0 |
| 9R-BA | 0.40 | 0.15 |
| DHZR | 100 | 0.85 |
| ZR | 1.0 | 100 |

as did DHZR and ZR (Table 1). The two bases and ribosides in the DHZ/Z and the DHZR/ZR fractions were measured with the appropriate antibody after the fractions were divided into two parts. Since the cross-reactivity of the ZR-antibody with DHZ and DHZR and also the cross-reactivity of the DHZR-antibody with Z and ZR were maximally 2% (Table 2), the amounts of all of these compounds could be accurately determined. BA and its riboside were separated by TLC from these natural cytokinins and, therefore, do not interfere with measurement of the natural cytokinins.

## *Physiological activity of various cytokinins*

No flower buds are formed on pedicel explants when cytokinin is omitted from the medium. The minimal concentrations of DHZ, Z, IP and BA to be applied for maximal flower bud formation *in vitro* were established (Tab. 3). The maximal number of flower buds formed varied between 12 and 14. The natural cytokinins as well as the synthetic cytokinin (BA) induced flower buds, beit at different concentrations. BA and DHZ induced maximal bud formation at 1 $\mu$M. A 20 to 40 times higher concentration of IP was required to evoke the same response. Z induced only one flower bud per explant, whereas Z concentrations higher than 10 $\mu$M were toxic.

The failure of explants to form buds in the absence of a cytokinin source in the medium demonstrates that the endogenous cytokinin concentrations are by themselves insufficient to induce bud formation. However, they might contribute to

Table 3. Effect of cytokinins on flower bud formation *in vitro* The concentration that induces maximal bud formation of four different cytokinins was established on MS medium in combination with 125 mM glucose and $\frac{1}{2}\mu$M NAA

| Cytokinin | concentration for max. bud formation ($\mu$M) | number of buds formed |
|---|---|---|
| BA | 1 | 12 |
| DHZ | 1 | 12 |
| IP | 20–40 | 14 |
| Z | 1–10 | 1 |

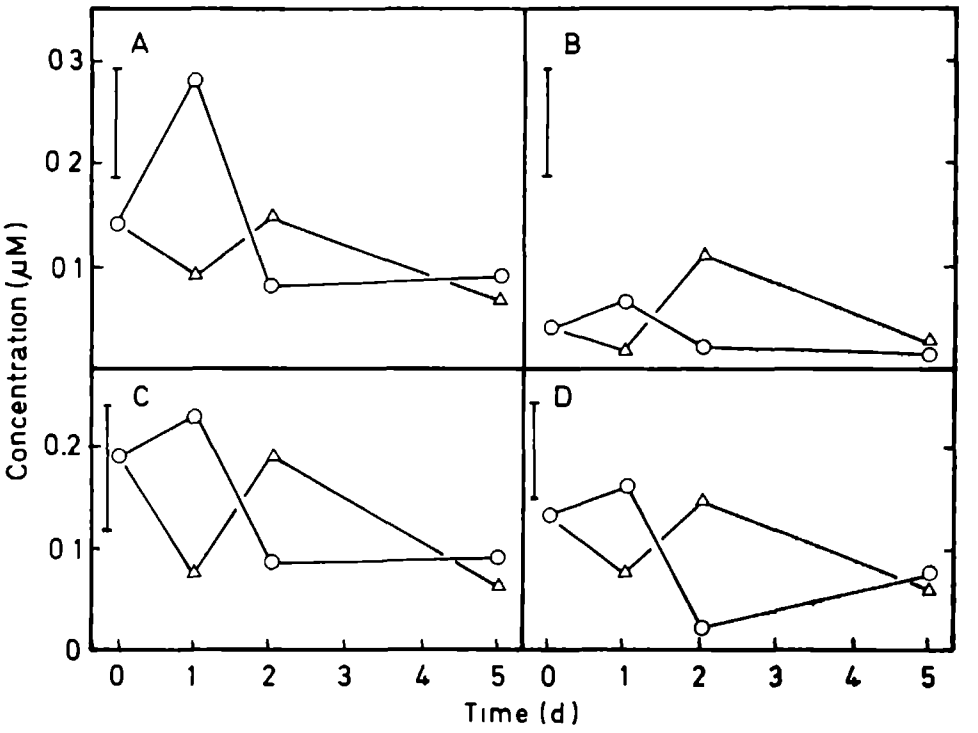


Fig 1 Concentration of endogenously synthesized cytokinin during the early phase of flower bud formation The DHZ (A) DHZR (B) Z (C) and ZR (D) concentration were measured with an ELISA in explants incubated on MS medium supplemented with glucose NAA and BA (Δ), the same medium without BA (○) The values are means of triplicate experiments Vertical bar LSD (p = 0 05)

the inductive cytokinin level in the explants Therefore, the concentrations of two endogenous cytokinins plus ribosides, one very active (DHZ) and one almost inactive (Z) with respect to flower bud formation, were measured. Explants were cultured on inductive and non-inductive media MS medium with BA and the same medium without BA (Fig 2a/d) The cytokinin concentrations in the explants at the beginning of the incubation were approximately 0 2 μM for DHZ and Z. The concentration of the ribosides was approximately half the concentration of the bases Explants incubated on non-inductive medium without BA showed an increase in the DHZ concentration after one day of incubation In contrast, explants incubated on inductive medium with BA had decreased in endogenous cytokinin concentration after 1 d of culture After 2 d of culture the concentration of DHZ was approximately the same as it was immediately after cutting of the explants and remained stable afterwards All changes in Z and their ribosides were small and not

significant

### *Contribution of endogenous cytokinins to the total cytokinin pool*

When DHZ is used to induce flower bud formation, the inductive concentration in the tissue is built up from endogenous synthesis as well as from uptake from the medium The role of endogenously synthesized DHZ in flower bud formation can be assessed by determining its contribution to the total amount of cytokinin present in the tissue To quantify the level resulting from uptake we measured DHZ uptake and conjugation during the 4-day induction period It was assumed that DHZ exerted the same influence on early DHZ synthesis as BA (Fig 2A) If so, the total level of DHZ sufficient for bud formation can be calculated The concentrations of the free BA or DHZ base in the explants derived from uptake from the medium is about 0 5 to 0 8 $\mu$M throughout the first four days of culture (Table 4). Since the endogenous DHZ level was 0 1 $\mu$M (Fig 2A), the total DHZ concentration

Table 4 The concentration of the free cytokinin base of BA and DHZ was determined after uptake during the early phase of flower bud formation Groups of 10 explants were incubated on MS medium supplemented with 125 mM glucose 1$\mu$M NAA and 1$\mu$M of [$^3$H]-BA or 1 $\mu$M [$^3$H]-DHZ Separation of the cytokinin base from its conjugates is described in materials and methods The values represent means of duplicate experiments

| Incubation time (d) | Uptake ($\mu$mol kg$^{-1}$) | | Free cytokinin base conc from uptake ($\mu$M) | |
|---|---|---|---|---|
| | BA | DHZ | BA | DHZ |
| 1 | 6 1 | 7 1 | 0 50 | 0 88 |
| 2 | 10 8 | 13 8 | 0 72 | 0 54 |
| 4 | 13 2 | 17 4 | 0 61 | 0 73 |

is 0 6 – 0 9$\mu$M

# DISCUSSION

Flower bud regeneration on pedicel explants of tobacco is depending on the cytokinin concentration in the tissue (Tran Thanh Van 1973, Van den Ende et al

1984). In *in vitro* cultured explants, the cytokinin concentration originates from uptake out of the medium and from endogenous synthesis. During tissue culture the endogenous DHZ concentration as measured by an ELISA technique was proven to be dependent on the cytokinin concentration in the medium. The endogenous cytokinins contributed 10–15% to the concentration optimal for flower bud induction.

The use of a Sep Pak $C_{18}$ cartridge and TLC for separation of BA, DHZ, Z and their ribosides proved to be a convenient method for purifying samples prior to quantification of these compounds by a competitive ELISA. The method was fast and after correction for losses during the procedure by means of the internal standard method, reproducible results could be obtained.

The explants used were peeled from the surface of the pedicels and therefore have a large wound area. Presumably as an result of wounding the concentration of DHZ temporarily increased from 0.15 to 0.3 $\mu$M in explants incubated on media without BA (Fig. 2 A–D). An increase in the endogenous cytokinin concentration after wounding was also found in cucumber cotyledons (Crane and Ross 1986) and oat leaves (Giridhar and Thiman 1985).

BA in the medium repressed DHZ accumulation (Fig. 2 A–D). The decrease was more than compensated by uptake. Despite extensive conjugation, explants incubated on 1$\mu$M DHZ contained approximately 0.8$\mu$M DHZ derived from uptake, which is three to five times more than in explants cultured in the absence of exogenous cytokinins. In the presence of an inductive BA concentration the level of DHZ is about 0.1$\mu$M (Fig. 2A). If DHZ provided in the medium is as active as BA in repressing endogenous DHZ accumulation, endogenous synthesis would contribute 10–15% to the total DHZ pool. This would mean that the role of endogenous DHZ in bud induction is modest. Presumably, endogenous Z is even less involved in flower bud formation since over the whole range of non-toxic medium concentrations maximally one flower bud per explant was induced (Table 3).

*Acknowledgement* – The authors are grateful to Mr CR Vonk for his gift of the antibodies.

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# PART 3: CYTOKININ-RELATED GENE EXPRESSION DURING FLOWER BUD FORMATION IN VITRO

# CHAPTER 7

## Cytokinin-related gene expression during bud regeneration in vitro in tobacco

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Submitted for publication.

# Cytokinin-related gene expression during bud regeneration *in vitro* in tobacco


## ABSTRACT

During bud formation *in vitro*, the effect of cytokinins, either endogenously synthesized or taken up from the medium, on the expression of the genes encoding for extensine, chitinase and one unknown protein was studied. The gene expression was investigated in pedicel and stem explants. The number of flower or vegetative buds formed was maximal at a medium concentration of 1 $\mu$M benzyladenine. No buds were formed after incubation on medium without the cytokinin. After one day of culture the total cytokinin base concentration in explants incubated on medium with or without benzyladenine was almost equal due to a high endogenous synthesis of cytokinins in explants incubated on medium without benzyladenine. The expression of the genes encoding for extensin and chitinase was cytokinin-related since their expression increased with an increase of the cytokinin concentration in the explants. The gene expression pattern is not related to bud formation because it occurs in all explants regardless of bud formation. The expression of the gene for the unknown protein did not change during culture, and was therefore not cytokinin-related. *In situ* hybridization data also showed no correlation between the expression of these genes and flower bud formation. The expression of the genes predominantly occurred in dividing cells, and was not resticted to meristems that develop into buds.


*Abbreviations:* BA, N6-benzyladenine; dCTP, deoxy-cytidine triphosphate; DHZ, dihydrozeatin; NAA, 1-naphthaleneacetic acid; DHZR, DL- dihydrozeatin-riboside; ELISA, enzyme-linked immunosorbent assay; LSD, least significant difference; 9R-BA, N6-benzyladenosine; TLC, thin-layer chromatography; Z, zeatin; ZR, zeatin-riboside.

## INTRODUCTION

Auxin and cytokinin induce regeneration of roots and shoots (Skoog and Miller 1957) or flower buds (Tran Thanh Van 1981) in tissue explants of tobacco. The general theory about hormone action is that a hormone induces the expression of primary target genes (Lewin 1987). The products of these genes can regulate other

genes and eventually lead to a cascade of effects. Especially in the case of cytokinins very almost nothing is known about the mode of action at the molecular level. There are a few reports on cytokinin-mediated changes in mRNA populations. Cytokinins were found to enhance the accumulation of nucleus-encoded mRNAs for chloroplast proteins in tobacco suspension culture (Teyssendier de la Serve et al. 1985) and Lemna gibba (Flores and Tobin 1986). Cytokinins were found to reduce the mRNA levels of some defence related proteins in tobacco (Mohnen et al. 1985, Shinshi et al. 1987). In contrast, in tobacco shoots exposed to elevated cytokinin levels either due to transformation with an active T-*cyt* gene, or to application of cytokinins in the culture medium, an increase in the mRNA levels related to this class of proteins was found (Memelink et al. 1987). Unfortunately, in none of these systems, the actual cytokinin concentration in the tissue was measured. Therefore, a quantitative relation between cytokinins and the regulation of genes has not yet been established.

In tissue culture, the cytokinin in the tissue is derived from hormone uptake from the medium and from endogenous synthesis. In determining the active cytokinin concentration, inactivation of part of the hormone by conjugation must be taken into account (Laloue and Pethe 1982, Van der Krieken et al. 1990).

The system of flower bud formation on pedicel explants (Tran Thanh Van 1973), is suitable to study the effect of cytokinins on gene expression in relation to the regeneration of buds *in vitro*. The concentration of the cytokinin in the medium determines the number of buds regenerated in this system (Van der Krieken et al. 1988). Uptake and conjugation of exogenously supplied cytokinin has been described (Letham and Palni 1983, Van der Krieken et al. 1990) and the endogenous cytokinin level can be quantified (Vonk et al. 1986). Since a large proportion of the cells in the explants participates in bud formation, changes in gene expression related to bud formation should be detectable.

In this paper we describe the relation between cytokinin concentration, the expression of three genes which have been described as cytokinin-related. Memelink et al. 1987) and bud formation. The concentrations of the active cytokinin bases derived from the medium and endogenously synthesized were measured. Since organized plant parts consist of different cell types, specific gene expression may be masked by the bulk of cells that do not respond to cytokinin. Therefore the expression of two of these genes was localized by *in situ* hybridization..

# MATERIAL AND METHODS

## *Culture in vitro*

Flowers of tobacco (*Nicotiana tabacum* L. cv. Samsun) were picked with pedicels attached at anthesis when the inflorescence was in full bloom (stage IV, Croes et al. 1985). Small explants (8x1 mm) were cut from the flower stalks and cultured on Murashige and Skoog (1962) medium with 125 mM glucose and 1% agar, according to Van den Ende et al. (1984). Explants of the same dimensions were also peeled from the stems of the top two internodes of 6 weeks old plants. 1-Naphthalene-acetic acid (NAA) was added at a concentration of 1$\mu$M BA was added at a concentration of 1 $\mu$M, or was omitted from the medium. To study uptake and conjugation, [2,8-$^3$H] BA (specific activity 15.5 TBq mol$^{-1}$, Amersham) was included.

## *Purification of the samples for ELISA*

Cytokinins were extracted from about 0.7 g of explants with 8 ml modified Bieleski fixative according to Laloue and Pethe (1982). At the beginning of the extraction $10^6$ dpm of [$^3$H]BA and [$^3$H]9R-BA (Amersham), specific activity 15.5 TBq mol$^{-1}$ and 20 TBq mol$^{-1}$ respectively, were added for determination of the recovery of the cytokinins. After prepurification by elution from Sep Pak $C_{18}$ cartridges (Waters), the samples were purified by thin-layer chromatography on silica gel C60 $PF_{254}$ plates Merck). On separate lanes authentic BA, 9R-BA, Z, ZR, DHZ and DHZR were added. The solvent used was *n*-butanol: 14 M $NH_4OH$: water (6:1:2 v/v/v), upper phase (Tao et al. 1983). The separated cytokinins were extracted from the silica and quantified with an ELISA test. For recovery calculations, the radioactivity in BA and 9R-BA was determined. The silica was scraped off and extracted with 0.5 ml water for 2 h. After addition of 4 ml Lumagel (Lumac), radioactivity was measured by liquid scintillation counting.

## *Enzyme-linked immunosorbent assay*

An indirect competitive ELISA was performed according to Vonk et al. (1986). Dynatech microtiter plates with high protein binding capacity were used. Polyclonal antibodies against ZR and DHZR and the protein conjugates of ZR and DHZR were

a generous gift of Mr CR Vonk.

## *Uptake and metabolism of BA*

For the determination of the uptake and conjugation of BA, groups of ten explants were cultured on [$^3$H] BA. After culture the explants were homogenized in modified Bieleski fixative (Laloue and Pethe 1982). The homogenate was concentrated and subjected to thin layer chromatography (Van der Krieken et al. 1988). The spots were scraped off and radioactivity was measured by liquid scintillation counting.

## *DNA probes*

The probes used to quantify gene expression are listed in Table 1. The pCNT probes were a kind gift of Dr. J. Memelink.

Table 1. The cDNA copies used in this study and their corresponding genes. The cDNA were cloned in pUN 121 (Memelink et al. 1987).

| cDNA clone | Characteristic for |
|---|---|
| pCNT 1 | extensin |
| pCNT 2 | unidentified protein |
| pCNT 4 | chitinase |
| pBR 21 | 18S and 28S ribosomal RNA |

## *Northern blot analysis*

Total RNA was isolated from the tissue by a modification of the method of Frankis and Mascarenhas (1980). 50 mM 2-mercapoethanol and 1% (w/v) tri-isopropyl-naphthalenesulfonic acid (Kodak) were added to the extraction buffer (Goldberg 1980).

Twenty $\mu$g of total RNA and lambda marker number III (Boehringer) were electrophorezed in a denaturing agarose gel according to Maniatis et al. (1982). After electrophoresis the RNA was blotted on a Hybond-N filter (Amersham) according to the instructions by the manufacturer. The RNA was covalently

cross-linked to the membrane with UV light for 5 min (Khandjian 1987).

The probes were random primed labeled together with the lambda marker with [$^{32}$P]-dCTP, specific activity 110 TBq mmol$^{-1}$ (Amersham) according to Feinburg and Vogelstein (1983, 1984) After hybridization the blots were autoradiographed (Church and Gilbert 1984) at -80 °C on Kodak XAR film using an intensifying screen (Cronex Plus, Dupont). The time for development of the film was chosen so that only grey hybridization signals were obtained. These signals were scanned with a densitometer (Biorad) The hybridization signal of the rRNA probe was used to correct for differences in the amount of total RNA in the lanes. After dehybridization (Church and Gilbert 1984) the blots were re-used with other probes, the rRNA probe being the final one

## *Hybridization in situ*

*In situ* hybridization was carried out with both anti-sense and sense [$^{35}$S]-RNA probes of pCNT 2 and 4. The probes were prepared by recloning the inserts of these plasmids in the Eco R1 site of a Bluescript M13-KS$^{+}$ vector (Stratagene). Runoff transcripts were made from the Bam H1 or Hind III linearized recombinant vector with T3 or T7 RNA polymerase. After probe synthesis, the DNA template was degraded with RNAse-free DNAse (Boehringer), and the probes were alkali hydrolized to a length of approximately 250 base pairs (Cox et al 1984) DNA and unincorporated nucleotides were removed using a G-50 spun-column Maniatis et al. 1982).

For tissue preparation, tobacco explants were fixed in 0 25% glutaraldehyde and 4% paraformaldehyde in 0 1 M phosphate buffer (pH = 6.8) for 3 h at 0°C. The tissues were dehydrated in graded series of ethanol and embedded in paraffin (mp 57 - 60 °C). Sections of 10 $\mu$m were cut and mounted on Tespa (Fluka) precoated microscope slides After removal of the paraffin with xylene, the sections were rehydrated and prepared for hybridization The method of Angerer and Angerer (1987) was used with some modifications. the length of the proteinase-K treatment was reduced to 10 min, and the acetic acid anhydride wash was replaced by a wash with 0.1 M ethanolamine for 10 min After dehydration in ethanol *in situ* hybridization was performed at 45 °C for 16 h in the mixture described by Martineau and Taylor (1986) with addition of 50 U ml$^{-1}$ RNasin (Promega) and 60 mM DTT. Twenty $\mu$l containing 2.5 pmol of labeled probe (specific activiy. 660 TBq mmol$^{-1}$) was put on each slide carrying 5 to 10 sections and covered with an 18 x

18 mm coverslip. Evaporation was prevented by sealing the coverslips with a water–insoluable glue. After hybridization, the sections were washed according to Raghavan (1989), and the slides were dried and subjected to liquid emulsion autoradiography (Illford Nuclear research emulsion G5) for 4 d at 4 °C. Finally, the film was developed and the sections were stained with Giemsa before they were permanently mounted in Enthalan. The silver grains were observed through a light microscope, with bright field and dark field illumination.

# RESULTS

## *Total concentration of active cytokinins in the explants during culture*

Explants from pedicels exclusively regenerate flower buds when cultured *in vitro*. After incubation on medium with 1$\mu$M NAA and 1$\mu$M BA 12 flower buds are formed. Under the same conditions, only vegetative buds develop in the same numbers on tissue strips cut from the upper two internodia of vegetative plants. Bud formation occurs synchronously in the two types of tissue and is complete after 14 days. Incubation of these explants on medium without BA does not lead to bud regeneration The cytokinin concentration in the explants is built up from BA taken up and from endogenous cytokinin synthesis. BA taken up from the medium is partly inactivated by conjugation. The active BA base concentration in the explants was measured during the early phase of bud formation, and was found to be

Table 2 Cytokinin concentrations during the first 5 d of culture in pedicel explants incubated on 0 and 1 $\mu$M BA Cytokinins were extracted from explants incubated for 1 to 5 d on media with or without 1$\mu$M [$^3$H]BA DHZ and Z were analyzed by an enzyme-linked immunosorbent assay, BA by measurement of radioactivity The results are expressed in $\mu$M and are the means of triplicate experiments

| Cytokinin base concentration inside the explants after different culture times | BA medium concentration ($\mu$M) | |
|---|---|---|
| | 0 | 1 |
| BA, 1 – 5 d | 0 | 0.6 |
| BA+DHZ+Z, 1 d | 0.51 | 0 77 |
| BA+DHZ+Z, 2 – 5 d | 0.20 | 0.87 |

constant during the first five days of culture. The concentrations of endogenously synthesized Z and DHZ were quantified immunologically (Table 2). One day after cutting the explants, the endogenous Z plus DHZ concentration in explants incubated on medium without BA is about three times higher than in explants incubated on 1$\mu$M BA. This led to a difference between bud forming explants and not-regenerating controls of 0.25$\mu$M in the total cytokinin concentration after 1 d. After 2 d this difference has increased almost three times to a constant value of 0.7 $\mu$M. The DHZ concentrations were almost equal to the Z concentrations in all samples and formed a constant fraction from 2 to 5 d of culture.

## *Quantification of hybridization signals*

For a good comparison of the hybridization signals on the blots, equal amounts of RNA in the different lanes are necessary. The amount of total RNA extracted from the explants was measured by UV-absorbance. In each lane on a gel about 20 $\mu$g

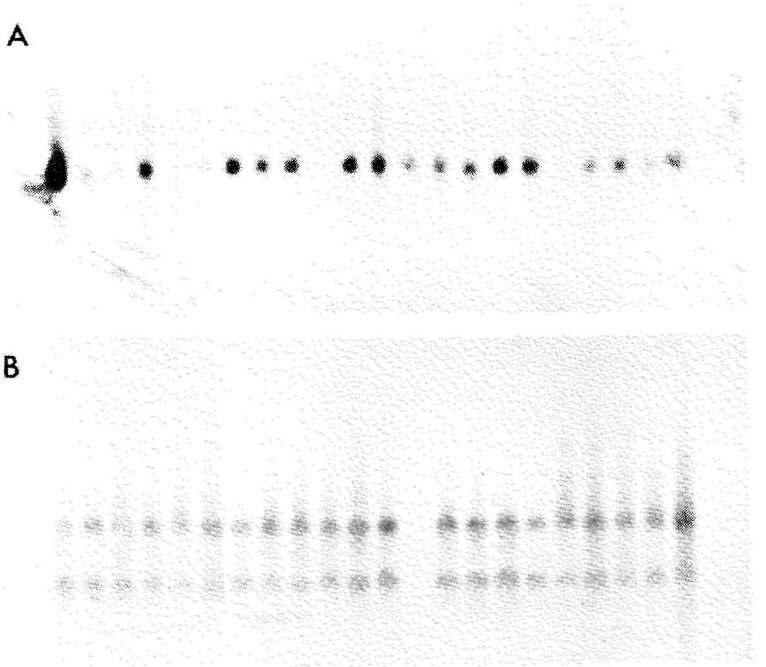


Fig. 1 (A-B). Northern blot hybridizations of total RNA of generative or vegetative explants incubated 0, 2, 4 or 14 d on medium with or without BA with [$^{32}$P]dCTP labeled probes of pCNT 2 and pBR 21 (Tab. 1).

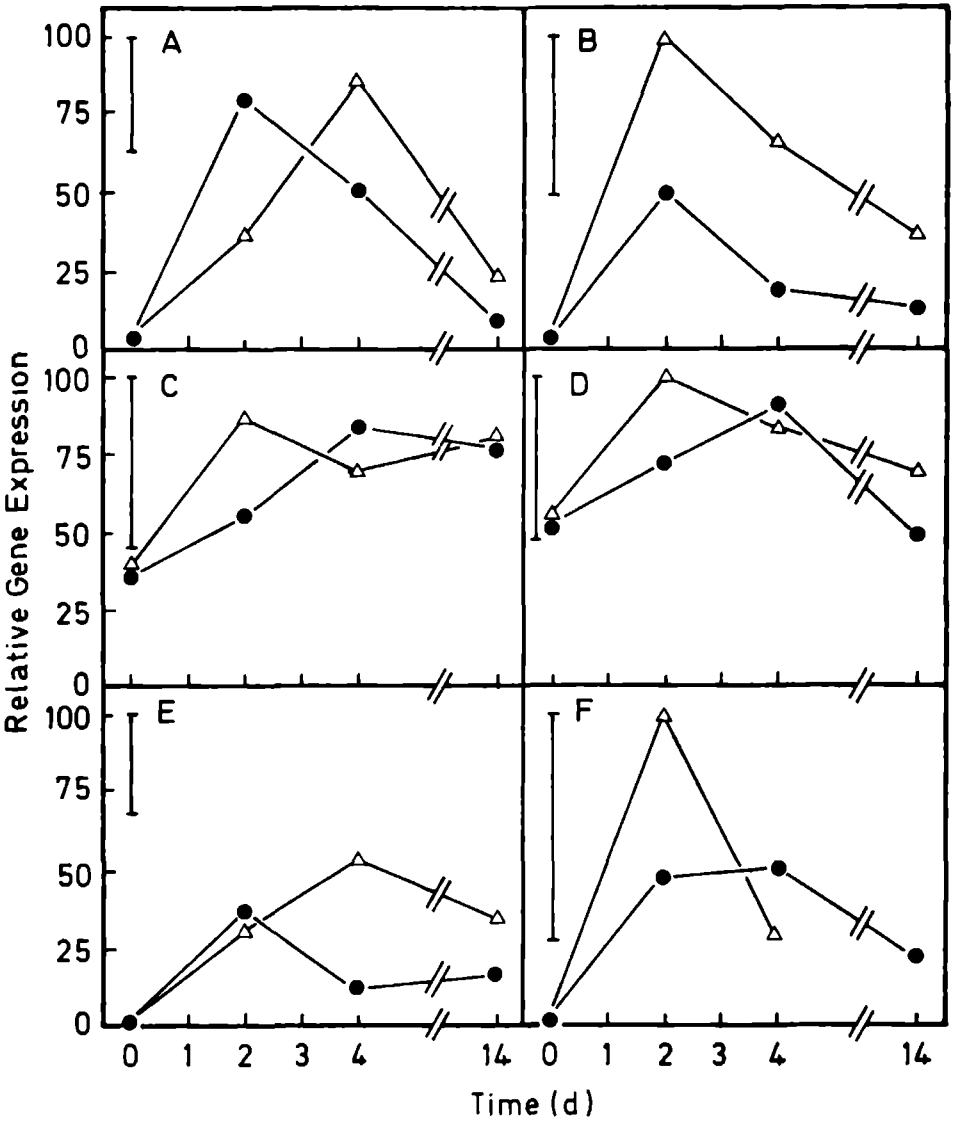


Fig 2 (A-F) Relative gene expression of pCNT 1 2 and 4 The gene expression was assessed during generative and vegetative bud formation in explants incubated on 1μM BA (●) or no BA (△) A, pCNT 1 generative, B pCNT 1 vegetative, C pCNT 2 generative, D, pCNT 2 vegetative, E, pCNT 4 generative, F pCNT 4 vegetative The hybridization signals were expressed as a percentage of the highest hybridization signal obtained with a particular probe The samples of fig A/B, C/D and E/F were investigated on the same blot The values are means of triplicate experiments Vertical bar: LSD (p = 0 05)

was separated. After blotting, the hybridization signals were quantified as described in Materials and Methods. The data were corrected for differences in hybridization of RNA in the same lanes with the rRNA probe (Fig. 1). This was done to overcome inaccuracies due to differences either in the amount of RNA in the lanes on a gel, or in the efficiency of blotting RNA to the filter. The results of the rRNA hybridizations revealed that in some lanes the amount of RNA was twice of that in other lanes.

## *Gene expression during bud formation in vitro*

The effect of the cytokinin concentration on the expression of cytokinin-related genes was studied during early bud initiation in generative and vegetative explants. Tissues cultured without BA served as a control. Gene expression was monitored by hybridizing total RNA from these explants to the probes pCNT 1, pCNT 4 and pCNT 2 (Table 1, Fig. 2 A–F). In all incubations, the hybridization signals to the

Table 3 Relative gene expression related to pCNT 2 and pCNT 4 in various cell types of pedicel explants after 0 and 4 days of culture on medium with or without 1 $\mu$M BA. Location of tissues, see Fig. 3. The values are means of 5 cells ± SE (in brackets). nm = not measureable because cell types do not exist in particular explant.

| Cell type | Probe | |
|---|---|---|
| | pCNT 2 | pCNT 4 |
| freshly cut explants | | |
| epidermis | 100 ± 12.4 | 76 ± 14 |
| cortex | 98 ± 5.7 | 100 ± 18 |
| 4 days old explants | | |
| | 1 $\mu$M BA | no BA |
| | pCNT 2 / pCNT 4 | pCNT 2 / pCNT 4 |
| epidermis | 32 ± 3.4 / 43 ± 4.3 | 47 ± 2.8 / 12.6 ± 1.6 |
| cortex | 31 ± 3.1 / 35 ± 4.0 | 59 ± 2.0 / 42 ± 4.8 |
| explant-side meristem | nm / nm | 100 ± 5.7 / 19 ± 2.9 |
| cells on medium | 100 ± 3.1 / 100 ± 4.6 | nm / nm |
| | 90 ± 4.1 / 40 ± 4.4 | 76 ± 2.3 / 100 ± 7.4 |

pCNT 1 and 4 probes were low immediately after start of culture in both types of explants. During the early phase of culture hybridization signals related to these probes increased significantly. After 14 d of incubation the gene expression is again low. The invariance of the response shows that the expression pattern is not influenced by the presence of cytokinin in the medium. The hybridization signal of pCNT 2 was approximately at the same level throughout culture in both types of explants (Fig. 2C–D).

## *Localization of gene expression by in situ hybridization*

After cutting, the explants consist of an epidermis layer and 4 or 5 layers of cortex cells. Culture for four days on medium with BA triggers cell division in the cells that are in contact with the medium and subsequently leads to the formation of meristems in the cortex (Fig. 3; Wilms and Sassen 1987). The structural diversity

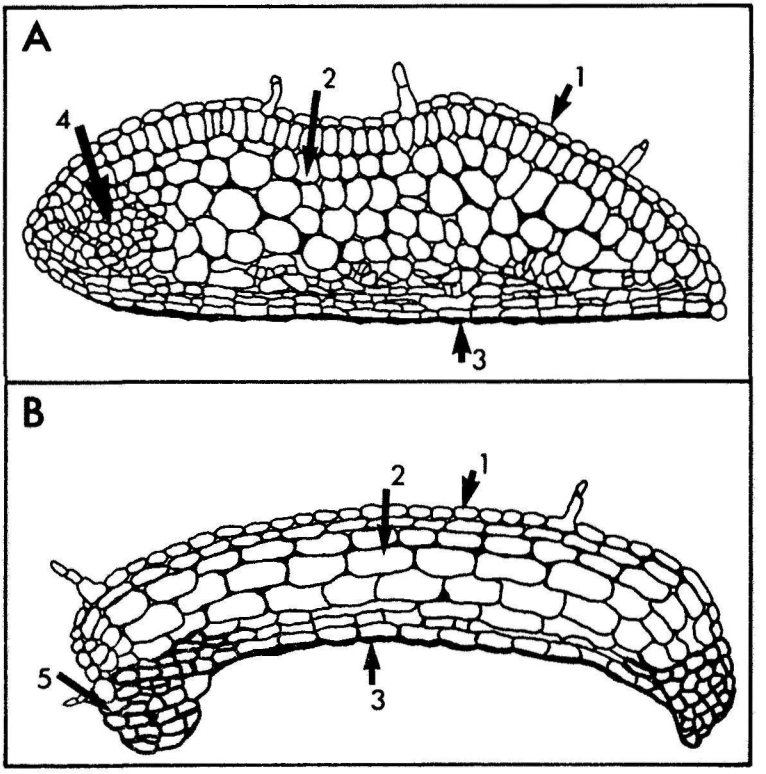


Fig. 3(A–B). Median section of an explant incubated on 1$\mu$M BA (A), or no BA (B). 1 = epidermis; 2 = cortex; 3 = cells in contact with medium; 4 = meristematic cells; 5 = cells at explant edge.

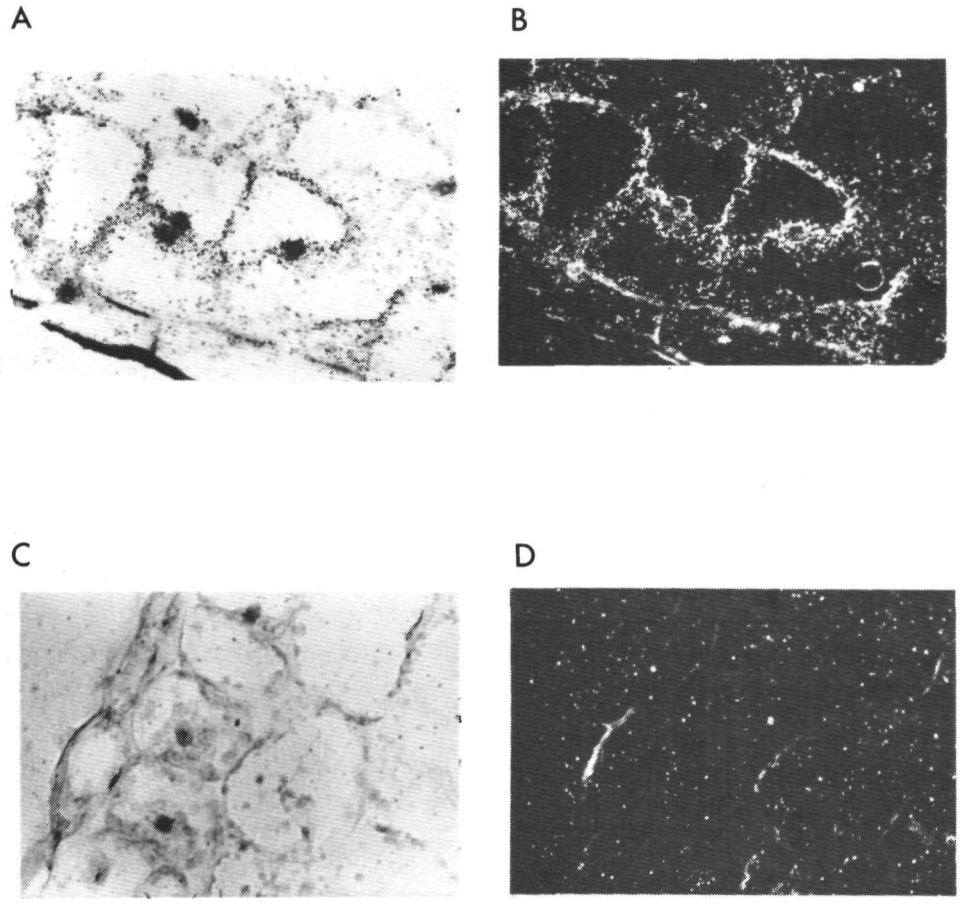


Fig. 4 (A–D). *In situ hybridization* of a freshly cut explant by assymetric [$^{35}$S]RNA probes, viewed by bright (A and C) and dark field (B and D) illumination. A and B hybridization with an anti-sense pCNT 2 probe; C and D hybridization with a sense pCNT 4 probe.

brought about by these activities might be associated with differential gene expression in the various cell types. To check this, the expression of the genes corresponding to the cytokinin related clones pCNT 2 and 4 was localized by *in situ* hybridization in median sections. The *in situ* hybridization was performed with anti-sense and sense [$^{35}$S] RNA probes. The sections tested with these probes were cut from the same explant. Since the mRNA accumulated predominantly around the nucleus the hybridization signal was counted in cells with a clearly visuable nucleus (Fig. 4). The relative distributions of the grains over the various cell types within one explant were compared after substraction of the non-specific hybridization signal of the sense probe (Tab. 3). Directly after cutting the explants, the mRNAs complementary to pCNT 2 and 4 are equally abundant in cortex and epidermis cells. After incubation for four days on medium with BA, hybridization with both RNA probes predominantly occurred in the meristematic cells from which the buds eventually develop and in the cell layer that is in contact with the medium (Fig. 3A). Incubation on medium without BA led for pCNT 2 to a slightly elevated hybridization level in the small cells at the edges of the explant, whereas pCNT 4 hybridized predominantly in the cells in contact with the medium (Fig. 3B).

# DISCUSSION

Cytokinins induce flower bud formation *in vitro* in pedicel explants of tobacco (Tran Thanh Van 1973). Therefore this system was used to study the expression of a set of cytokinin-related genes to see whether such genes can be used as marker genes for cytokinin induced differentiation processes in plants. The expression of two of these genes followed a rise of the endogenous cytokinin concentration in the tissue, and occured regardless whether or not buds were formed. *In situ* hybridization data showed that gene expression was predominantly localized in dividing cells.

The strength of the hybridization signal obtained from the rRNA probe is a measure of the amount of mRNA in a lane of a blot. This is based on the assumption that the ratio between mRNA and rRNA is approximately constant. The use of the rRNA probe, therefore, enabled us to compare the expression of specific probes in different tissues on basis of equal amounts of mRNA.

The expression of two of the genes under study (probes pCNT 1 and 4) is preceded by a rise in the cytokinin concentration in the tissue (Fig. 2, Tab. 2). In bud forming explants the increase during the first day of culture was a result of BA

uptake (Van der Krieken et al. 1988). Little DHZ and Z accumulated in the presence of BA (Table 2). Tissues incubated without BA endogenously synthesized cytokinins to a level that is approximately equal to the total cytokinin concentration in BA treated explants in the same period (Table 2). This endogenous synthesis is presumably a response to wounding (Crane and Ross 1986). The correlation between gene expression and cytokinin level holds for both vegetative and generative explants and is, at least in generative explants, independent of the presence of a cytokinin in the medium (Table 2). The pattern of expression of these genes coding for chitinase (pCNT 4) and extensin (pCNT 1) is, therefore, in line with their expression in tobacco shoots treated with cytokinin or in transgenic shoots carrying the isopentenyl–transferase gene of *Agrobacterium tumefaciens* (Memelink et al. 1987). However, there was no relation between endogenous cytokinin concentration and expression of the third gene investigated (pCNT 2). Although the expression of this gene has been described as cytokinin–related before (Memelink et al. 1987), the evidence for this is not unequivocal because the cytokinin concentration in the material used in that study has not been determined. The expression pattern of this gene might be affected by tissue culture conditions.

The genes corresponding to pCNT 2 and 4 are expressed in all cell types of the explant to different extents. After four days of culture (Tab. 3), activities were associated with the meristems and the cells in contact with the medium, tissues which are actively dividing. The correlation between cell division and expression is more pronounced in the case of pCNT 4 than with pCNT 2, where the expression pattern is more diffuse. Since cytokinins are known to play a role in cell division, the predominant expression of pCNT 4 in dividing cells is an extra argument for its relation with cytokinin. At present, it is not yet known when during regeneration flower bud specific processes occur for the first time. The similarity in expression of the genes studied in vegetative and generative explants argues against a specific role of these genes in flower bud formation. However, expression of cytokinin related genes may be a marker for cell division and meristem organization, processes which precede flower bud formation. Such a possibility is in particular open for the extensin gene (pCNT 1) which may function in early cell division.

Current research is now focussed on the isolation of genes directly related to flower bud formation from this tissue culture system (Peters, in preparation).

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# CHAPTER 8

## Algemene Samenvatting

# ALGEMENE SAMENVATTING

Het in dit proefschrift beschreven onderzoek behandelt de rol van cytokininen bij de regulatie van bloemknopvorming *in vitro* in dunnelaag-weefselkweekstrips van tabak. Hierbij is in het bijzonder de rol van het metabolisme, de endogene synthese en de genexpressie bestudeerd.

## METABOLISME EN BLOEMKNOPVORMING *IN VITRO*

Op explantaten van bloemstelen van tabak onstaan bloemknoppen na incubatie op medium met voedingszouten, sucrose en de plantenhormonen auxine en cytokinine. Het aantal bloemknoppen dat gevormd wordt hangt af van zowel de auxine- als de cytokinine-concentratie in het weefsel. Als één van beide hormonen in voldoende mate aanwezig is, dan is met het andere hormoon een bloemknopaantal varierend van 0 tot ongeveer 12 induceerbaar (hoofdstuk 2). Niet alle cytokininen zijn even actief in het induceren van de bloemknoppen. Dihydrozeatine (DHZ) induceert het maximale aantal bloemknoppen bij de laagste concentratie in het medium. Isopentenyladenine (IP) is 20 tot 40 maal minder actief en zeatine (Z) leidt slechts tot de vorming van maximaal één bloemknop. Het synthetische benzyladenine (BA) vormt het maximale aantal bloemknoppen bij dezelfde mediumconcentratie als DHZ. (hoofdstuk 3). Bekend is dat cytokininen gemetaboliseerd worden na opname door plantenweefsel (Letham and Palni 1983). In de tabaksexplantaten wordt BA gemetaboliseerd tot glucosiden, het 9-riboside en de mono-, di-, en trifosfaten (nucleotiden). DHZ wordt naast deze conjugaten ook nog omgezet in een O-glucoside, en bij IP treedt ook afbraak van het hormoon op (hoofdstuk 2, 3, 4, 5).

Op verschillende manieren is onderzocht welke metabolieten actief zijn wat betreft bloemknopvorming. Het metabolisme van cytokininen is bestudeerd in explantaten die geïncubeerd zijn op inductieve en niet-inductieve concentraties voor bloemknopvorming (hoofdstuk 2, 3, 4, 5). Ook het verloop van het metabolisme op verschillende tijden gedurende bloemknopvorming is bestudeerd (hoofdstuk 4). Het metabolisme is echter onder alle geteste omstandigheden constant, zodat geconcludeerd kan worden dat het metabolisme niet specifiek is voor bloemknopvorming. Een manier om toch de activiteit van de cytokinine-metabolieten bij bloemknopvorming te bepalen is het gebruik van een relatief

inactief cytokinine voor bloemknopvorming (IP) als competitieve inhibitor van het metabolisme van een zeer actief cytokinine (BA) Explantaten geincubeerd op inactieve IP-concentraties in combinatie met niet-optimale BA-concentraties vormen meer bloemknoppen dan explantaten geincubeerd op alleen BA Deze toename gaat gepaard met veranderingen in BA-opname en -metabolisme De opname van BA wordt lager maar door een sterk gereduceerd metabolisme is toch de concentratie van de BA-base in de explantaten significant hoger Door de remming van de omzetting van BA is de concentratie van het BA-glucoside veel lager, terwijl die van het riboside en de nucleotiden ongeveer gelijk blijkt Omgekeerd is het effect van BA op het IP-metabolisme zodanig dat IP of zijn metabolieten niet verantwoordelijk kunnen zijn voor de toename in het bloemknopaantal Uit dit alles blijkt dat de cytokinine-base fysiologisch actief is, maar activiteit van de nucleotiden en ribosiden kan niet volledig worden uitgesloten (hoofdstuk 3)

## *ENDOGENE CYTOKININEN EN BLOEMKNOPVORMING*

Een totaal beeld van de rol van cytokininen bij de bloemknopvorming wordt verkregen als naast de cytokinine-concentratie afkomstig uit het kweekmedium ook de concentratie van de endogeen gesynthetiseerde cytokininen bepaald wordt In dit proefschrift wordt de concentratie van endogeen gesynthetiseerd DHZ en Z en hun ribosiden beschreven Door technische moeilijkheden wordt de concentratie van het relatief inactieve IP en en zijn riboside niet bepaald In vers gesneden explantaten die geincubeerd worden op medium zonder cytokininen of zonder koolstofbron, neemt als gevolg van de reactie op de toegebrachte wond de endogene cytokinine-inhoud tijdelijk sterk toe Echter, in explantaten geincubeerd op medium met cytokininen wordt een tijdelijke afname gevonden van de endogene cytokininepool Gedurende de initiatieperiode voor de bloemknopvorming is de concentratie van de fysiologisch actieve DHZ-base ongeveer 25% van de uit het medium opgenomen BA-base Hieruit blijkt dat de endogene cytokininen hoogstwaarschijnlijk een ondergeschikte rol spelen voor de bloemknopvorming *in vitro*

## *CYTOKININE GEREGULEERDE GENEXPRESSIE*

Uit het voorgaande onderzoek is bekend wat de totale concentratie van de actieve cytokinine basen, die leidt tot maximale bloemknopvorming, is. Onderzocht wordt wat het effect is van cytokininen op de genregulatie die betrokken is bij de bloemknopvorming. Hiertoe wordt met behulp van cDNA-copieen het effect van inductieve en niet-inductieve cytokinine-concentraties voor bloemknopvorming op de expressie van drie cytokinine-specifieke genen onderzocht. Twee cDNA-copieen nl pCNT1 en pCNT4 corresponderen met twee infectie-gerelateerde eiwitten, respectivelijk extensine en chitinase; een derde cDNA cloon, pCNT2 met een onbekend eiwit . Tijdens de *in vitro* cultuur van de tabaksexplantaten blijkt dat de genexpressie behorende bij pCNT 1 en 4 toenam. Deze toename vindt plaats onafhankelijk van de cytokinine-concentratie in het medium, en is dus niet gerelateerd aan bloemknopvorming. Om te bepalen of de expressie van deze genen cytokinine-specifiek is wordt de totale actieve cytokininenconcentratie in de explantaten gemeten. Zoals boven beschreven, neemt in explantaten geïncubeerd op medium zonder cytokininen de endogeen gesynthetiseerde cytokinineconcentratie toe, en in explantaten geïncubeerd op medium met cytokininen, neemt de concentratie af. Dit leidt tot een totale cytokinine-inhoud na één dag incubatie die ongeveer gelijk is ongeacht de mediumconcentratie. De genexpressie behorende bij pCNT 1 en 4 kan dus cytokinine-specifiek zijn. De genexpressie van pCNT 2 blijft op een constant niveau en was niet cytokinine-specifiek in de bloemsteelexplantaten.

In het vorige experiment werd de genexpressie bestudeerd aan de hand van de totale geëxtraheerde RNA-fractie afkomstig uit alle cellen van het explantaat. De genexpressie die specifiek in bepaalde weefseldelen verhoogd is, kan met deze methode niet gemeten worden door de grote hoeveelheden cellen die niet reageren op de toegediende cytokininen. Daarom wordt m.b.v. *in situ* hybridisatie de relatie tussen de genexpressie en de bloemknopvorming in detail bekeken. Dit wordt gedaan door te bestuderen of de genexpressie in meristeemcellen waaruit uiteindelijk de bloemknoppen ontstaan sterker was dan in andere celtypen. In dit onderzoek worden pCNT 2 en pCNT 4 gebruikt als probes. Na vier dagen incubatie op medium met cytokinine komen beide probes voornamelijk tot expressie in de meristemen waar uiteindelijk de bloemknoppen uit ontstaan. De probes komen echter ook sterk tot expressie in ander delend weefsel zoals in de cellen die in kontakt staan met het medium. Na incubatie van weefsel op medium met alleen auxine treedt een sterke expressie op in de delende cellen aan de basale zijde van

het explantaat. Specificiteit van de genexpressie voor bloemknopvorming is hierdoor niet waarschijnlijk.

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De afdelingen Illustratie en Fotografie hebben de figuren in dit proefschrift gemaakt.

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# CURRICULUM VITAE

Wim van der Krieken werd geboren op 13 december 1957 te Steensel. Het diploma Atheneum-B behaalde hij aan het Rythovius college te Eersel in 1976. In 1977 begon hij met de studie biologie aan de Katholieke Universiteit te Nijmegen. In april 1985 werd deze studie afgesloten met het behalen van het doctoraal examen met als hoofdvak Plantenfysiologie (Dr. G.J. de Klerk; Prof. Dr. H.F. Linskens) en bijvakken Microbiologie (Dr. L.G.M. Gorris; Prof. Dr. G.J. Vogels) en Toxicologie (Dr. R. Bos; Prof. Dr. P.Th Henderson). Vanaf augustus 1985 tot augustus 1989 was hij werkzaam als wetenschappelijk medewerker op de afdeling Moleculaire Plantenfysiologie aan de Katholieke Universiteit van Nijmegen. Onder leiding van Dr. A.F Croes, Dr. G.W.M. Barendse en Prof. Dr. G.J. Wullems werd het in dit proefschrift beschreven onderzoek verricht.

Vanaf oktober 1989 is hij werkzaam als wetenschappelijk medewerker bij het Centrum voor Agrobiologisch Onderzoek te Wageningen.

Op 23 augustus 1982 trouwde Wim met Desiree Engelen en ze hebben nu samen een dochter, Danique.